

CEREAL GRAIN BIOCHEMISTRY AND

RELATED STUDIES

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## ABSTRACT

The greater part of this work describes the biochemical and morphological changes accompanying grain development in cereals. Attention is focussed on those aspects most likely to be involved in the regulation of grain yield and composition. Thus, since starch is the major component of grain yield much of the work is concerned with the developmental enzymology of carbohydrate synthesis. The composition, properties and structure of the developing starch granules and amyloplasts have also been described. Grain development is associated with a range of catabolic processes and a number of enzymes active in carbohydrate degradation has been identified. Although oxygen levels in the developing endosperm are unknown, recent work suggests that the mitochondria present are fully functional. It is possible that oxygen required for grain respiration is derived from pericarp photosynthesis. At the same time this tissue may fix carbon dioxide derived from endosperm respiratory processes. Thus, the pericarp may be involved in the overall control of endosperm metabolic activity. Studies of endosperm cell division indicate that this continues for more than half the developmental period, later divisions producing mainly aleurone cells. Hence, stimulation of endosperm cell division early in development will increase the yield of carbohydrate whereas at later stages it will increase the yield of protein. Further studies describe the pattern of mineral ion accumulation, the growth and nutrition of the developing embryo and the morphological changes in the crease region through which nutrients move from the vascular tissue of the pericarp to the endosperm.

A second group of papers is concerned with the control of  $\alpha$ -amylase and polyphenol oxidase activity during cereal grain germination. Finally, there is a small group of papers describing research on the properties of photosynthesising tissues including marine algae.

## CEREAL GRAIN DEVELOPMENT

In this section the biochemical and physiological changes accompanying grain development in cereals are described, with the hope of identifying some of the factors concerned in the regulation of grain yield and composition. Clearly, other plant processes have a major influence on yield, however there seems little doubt that those most directly involved are those occurring in the grains themselves. Furthermore, there is some justification for concentrating on post-fertilisation events, since it seems likely that the dry matter entering the grain for subsequent storage is derived from photosynthesis taking place after ear emergence. It is even possible that, since cereal plants, under most environmental conditions may not operate at full photosynthetic capacity, events in the grain themselves may regulate photosynthesis and hence, indirectly, overall yield.

## Carbohydrate metabolism

In 1968, when this research programme was initiated, little was known of the biochemical, or even structural, changes accompanying grain development. One of the first publications<sup>4</sup> recorded the changes in activity of a number of enzymes thought to be concerned with amyloplast development. Although this work was useful in that it indicated that enzymes associated with oxidative processes were present, it did not make any attempt to differentiate between the different tissues of the grain or to present any data on starch granules or amyloplast development.

As a result of this early work it was realised that any study of grain development must take into account the morphologically distinct grain tissues and their genetic origins. For example, although the aleurone layer may be separated from the endosperm at certain stages of grain development, it is a part of the endosperm and should be considered with the starchy endosperm. The embryo in turn is

morphologically and genetically distinct and it too should be treated separately, care being taken to remove the surrounding layer of aleurone before investigation.

Since starch is the major component of grain yield, much of the work is concerned with the developmental enzymology of carbohydrate synthesis. Thus it has been shown that the UDP-dependent enzyme, sucrose synthase, probably plays a part in the sucrose-starch conversion<sup>9</sup>. Since one of the products of this reaction is UDP-glucose, but the nucleotide sugar donor for starch synthase is ADP-glucose<sup>5,10</sup>, a scheme was proposed<sup>9</sup> whereby a coupling of the UDP- and ADP-glucose pyrophosphorylases would allow a rapid conversion of sucrose to starch via ADP-glucose starch synthase. The fructose formed as a result of sucrose synthase activity may be converted to nucleotide sugars via fructokinase, phosphoglucose isomerase, phosphoglucomutase and UDP(ADP)-glucose pyrophosphorylase.

While the bulk of  $\alpha$ -(1,4) glucan synthesis is now generally considered to be catalysed by starch synthase, little is known of the factors involved in the initiation of starch synthesis or of the origin of the primers required for the starch synthase reaction. Certainly unprimed phosphorylase activity was present<sup>13</sup> early after fertilisation in barley endosperm extracts and may be associated with the initiation of primers for starch synthesis. This problem has not yet been resolved, partly due to difficulty in obtaining primer-free extracts.

On the basis of relative enzyme activities it was concluded<sup>30</sup> that ADPG-pyrophosphorylase may be the rate-limiting enzyme for starch synthesis.

#### Carbohydrate degradation

Subsequent work<sup>33</sup> has shown that development in cereal grains is accompanied by catabolic processes active in starch hydrolysis and degradation. This is not really surprising since energy is required

for the great variety of synthetic processes carried out by the endosperm cells during development. Both  $\alpha$ - and  $\beta$ - amylases are present<sup>2,8</sup> in immature barley grains. However they are unlikely to be involved in the degradation of immature starch granules<sup>20</sup>, and their role may be in the degradation of oligosaccharides. Both glycolytic<sup>19</sup> and mitochondrial<sup>4</sup> enzyme activities are present in developing barley endosperm. This, together with the possible association of mitochondria and amyloplasts,<sup>24</sup> suggests that active respiratory processes accompany grain development and may supply energy for starch and protein synthesis. The early appearance of a number of transaminases in developing endosperms<sup>25</sup> suggests an additional role for oxidative carbohydrate metabolism in the supply of carbon skeletons for amino acid and synthesis.

Accompanying carbohydrate and protein deposition in the developing grain is an overall accumulation of mineral elements. The role of these in the synthesis of grain storage material has been investigated both in relation to the essential major<sup>16</sup> and the essential trace<sup>17</sup> mineral elements. It was clear from this data that much variation existed between the different grain tissues and that mechanisms may exist whereby nutrients can be transferred from the pericarp to other parts of the grain.

#### The amyloplast

The role of the amyloplast is clearly central to any study of the regulation of starch synthesis. However it has not so far proved possible to isolate amyloplasts throughout the developmental period and demonstrate in every case the presence of an intact surrounding double membrane as seen in electron micrographs of fixed and embedded endosperm sections<sup>24,33</sup>. The isolated 'amyloplasts' referred to in some of the work reported here<sup>5,15</sup> are almost certainly a mixture of starch granules and amyloplasts. In later work such preparations are referred to as starch granules, although they doubtless contain a proportion of

amyloplasts. The starch granules of immature endosperm exist in a range of sizes, although in mature grains, as in potato tubers<sup>15</sup>, they are more readily divided into two distinct populations, large and small. By fractionating barley endosperm starch granules at different stages of development, labelled with  $^{14}\text{C}$  derived from flag leaf photosynthesis, it has been shown that the small amyloplasts are most likely initiated after about 2 weeks of endosperm development<sup>24</sup>.

The amount of amylose, relative to the amount of starch present, increases throughout grain development<sup>26,32</sup>. That this may be caused by changes in relative numbers of the different size classes present, which show increasing amylose content with increasing size, has been demonstrated<sup>20,26,32</sup> using starch granule fractions isolated from endosperms at different stages of development.

#### The embryo

Since my major interest is in the control of grain yield it was obviously of relevance to investigate processes taking place in tissues other than the endosperm. In particular it was important to define the nutritional requirements of the developing embryo as well as to establish the overall biochemical changes accompanying embryogenesis.

The cereal embryo lies at the base of the grain on the dorsal side and in barley grows to a maximum length of around 3.5 mm<sup>28</sup>.

The developmental pattern<sup>14</sup> of the enzymes involved in sucrose metabolism was very similar to that recorded previously<sup>9</sup> for the endosperm, but the presence of significant invertase activity suggested that in the early stages of embryo growth some sucrose is cleaved by invertase during entry and diffuses as monosaccharides to the embryo cells.

In order to study the growth requirements of embryos a system for their culture *in vitro* was devised<sup>23</sup>. It was shown that embryos are very exacting in their requirements. However a degree of biosynthetic

autonomy appears very early in development, since apparently normal growth could be sustained on a restricted growth medium containing a limited range of amino acids and sucrose, but a full spectrum of essential mineral elements. It was also observed in these and other<sup>35</sup> experiments that high sucrose concentrations may delay the onset of germination. This in turn may suggest that the phenomenon of pre-harvest sprouting, common in Northern Europe, South America and Canada, may be a function of endosperm osmotic potential and might be controlled by variation in concentration of some metabolite or metabolites. Further work<sup>28</sup> on the nutrition of isolated embryos indicates that active transport plays a major part in sucrose uptake. Hence this may suggest a need to compete with the endosperm for sucrose. On the other hand their dependence on passive diffusion<sup>31</sup> for the assimilation of monosaccharides is compatible with the relatively minor role of these sugars in embryo nutrition.

#### Photosynthesis and Grain Development

Cereal grains, with the exception of maize, are capable of light-dependent carbon dioxide fixation. This takes place in the chloroplast-containing cross cells of the pericarp<sup>11</sup>. Although this tissue may not have any of the morphological characteristics of  $C_4$  plants<sup>29</sup>, it has a number of enzymes<sup>11</sup> characteristic of  $C_4$  plants and, interestingly, the first-formed product of carbon fixation is malate<sup>18</sup>.

It may be that carbon dioxide can reach the green layer of the pericarp from the atmosphere, by diffusion through stomata on the surface of the pericarp epidermis<sup>29</sup>, as well as from the endosperm, by diffusion through the cuticular layers of the testa and nucleus. It may be then, that the pericarp functions in the re-fixation of endosperm carbon dioxide and thus helps to reduce respiratory losses. It seems likely that oxygen evolved during grain photosynthesis remains within the grain<sup>18,27</sup> and that this may be the source of oxygen for grain respiration. If this is the case, then the pericarp may be involved in



the control of the entire metabolic activity of the endosperm.

### Morphological changes and grain development

Until recently there have been few reports of the morphological changes accompanying grain development. This was partly due to difficulties in obtaining good sections of a tissue, which varies from a semi-liquid at early stages to a hard, somewhat flinty tissue of low moisture content at maturity.

Initially endosperm nuclei proliferate without cell wall formation. Thus it proved possible to isolate a relatively uncontaminated and intact nuclear fraction<sup>7</sup>. These are extremely fragile. They are metabolically active and are rather unusual in that they appear to have only one histone. Starch deposition within amyloplasts commences early in development, as does the formation of protein bodies<sup>33</sup>. Many of the mitochondria are grouped round the immature amyloplasts<sup>24</sup>.

In later work the morphology of the outer layers of immature cereal grains was considered<sup>29</sup> and the significance of the cuticular layers in relation to carbon dioxide and oxygen transport discussed (see above). These studies were then extended<sup>34</sup> to the nucellar projection and modified aleurone in the crease region of immature barley caryopses. These are of particular interest since they lie on either side of a gap in the route along which nutrients move from the vascular tissue of the pericarp to the developing endosperm. As observed by many other workers it seemed that one of the barriers to nutrient uptake is the lipid plug which forms between the ends of the testa in the nucellar projection.

Since grain weight is one of the major components of grain yield and since weight may be a function of cell number it was of interest to establish the stage of development at which maximum cell number is attained. The results<sup>37</sup> demonstrated that in barley, endosperm cell division continues for at least half of the period between anthesis and maturity, later divisions producing mainly aleurone cells. This

observation is of particular importance since stimulation of endosperm cell division early in development could increase the yield of carbohydrate whereas stimulation of cell division at a later stage could increase the yield of protein, lipid, vitamins and minerals which are found in aleurone cells.

To conclude, some of the factors responsible for the regulation of grain growth and development have been identified. These include the activity of the enzyme ADP-glucose pyrophosphorylase, the photosynthetic activity of the chloroplast-containing layer of the pericarp, the progressive formation of the lipid plug and the rate of endosperm cell division.

#### CONTROL OF GERMINATION

By 1968, cyclic adenosine monophosphate (cyclic-AMP) had been established as an intermediate in the action of a number of mammalian hormones. It was therefore of interest to determine whether or not it had a similar role in plants. It was found<sup>1</sup> that cyclic-AMP would stimulate  $\alpha$ -amylase release in barley endosperm slices, thus mimicking the effect of gibberellic acid. The effect, although significant, was small. Subsequent searches by other workers to identify cyclic-AMP in germinating barley have not been successful. Since prostaglandins may be concerned in the regulation of cyclic-AMP, their effect on the barley endosperm system was investigated<sup>12</sup>. While  $\alpha$ -amylase was released in response to prostaglandin  $F_{2\alpha}$ , neither  $E_1$ ,  $E_2$  nor  $A_1$  had any significant effect.

The presence of a gonadotrophin-like growth regulating factor has been reported in higher plants. Since gibberellin levels may be lowered in the presence of human chorionic gonadotrophin (HCG), it was proposed that this factor might be involved in controlling gibberellic acid synthesis. However, the exact significance of the evidence presented must be open to doubt since gibberellin activity was assayed simply by



release of reducing sugars. Furthermore HCG was found to inhibit gibberellic acid-triggered reducing sugar release. Our results showed that HCG inhibited gibberellin synthesis in barley embryos but had no effect on gibberellin transfer from the embryo. It did not affect the response of barley aleurone to gibberellin as measured by  $\alpha$ -amylase production<sup>3</sup>.

Further studies showed that as  $\alpha$ -amylase activity increased in barley aleurone cells during germination, glutamate decarboxylase activity decreased. Subsequently it was found that  $\gamma$ -aminobutyric acid (GABA) the product of the decarboxylase action, could inhibit  $\alpha$ -amylase activity. We therefore suggested<sup>6</sup> that this molecule might have a regulatory role in germination.

Gibberellic acid may also be involved in the regulation of polyphenol oxidase. The response of wheat and barley embryoless half grains to gibberellic acid was further investigated<sup>21</sup> in relation to o-diphenolase activity. It was found that gibberellic acid enhanced activity in wheat but inhibited it in barley. Subsequently<sup>22</sup> it was found that the increase in wheat o-diphenolase activity which appears in the absence of gibberellic acid and the activity promoted by gibberellic acid were dependent on protein synthesis.

The overall physiology and biochemistry of seeds is described<sup>36</sup> in the book 'Seeds and their Uses'.

#### RELATED STUDIES

The final group of papers includes several investigations of the properties of photosynthesising tissues. The first papers relate to the

metabolism of glycollic acid and describe work submitted for the Ph.D. degree at the University of London. At that time the site of origin and mechanism of formation of glycollate was unknown, and the results demonstrated quite clearly, using the then novel technique of chloroplast isolation on non-aqueous density gradients, that two of the enzymes possibly involved in glycollate formation were localised in the chloroplast<sup>38</sup>. One of these was phosphoglycollate phosphatase and it was then suggested that glycollate might be derived from a sugar diphosphate of the photosynthetic carbon reduction cycle.

Since glycollate oxidase activity was found to be absent from the chloroplast it was concluded<sup>39</sup> that further metabolism of glycollate takes place outside the chloroplast. It seemed likely, therefore, that at least under certain conditions, glycollate may act as a carrier exporting reducing equivalents or even carbon from the chloroplasts to the cytoplasm. Even now the role of glycollate in plant metabolism is largely unexplained, but it is generally agreed that glycollate originates from a sugar bisphosphate and is subsequently exported from the chloroplast.

The two final papers are concerned with the nutritional properties of marine algae.

The first<sup>40</sup> describes an investigation of some of the digestive enzymes present in coastal mollusca from the Canary Island of Lanzarote. Previous work had established a correlation between the mollusca and the benthic algae on which they were found. The object of this study was to assess the ability of the mollusca to use the algae as food. While firm conclusions could not be made it was interesting to note that cellulase was present in all species, with one exception, *Thais haemastoma*. Possibly this is a function of its carnivorous, rather than vegetarian, habit.

Further studies<sup>41</sup> with photosynthesising tissues have included an investigation of the nutritional properties of the marine alga

*Dunaliella*. The high protein content, favourable amino acid composition, low nucleic acid and cellulose contents suggest that *Dunaliella* is a potentially valuable food source. The high salt content of unwashed algal pellets prevents their use in animal diets at levels much above 10%. This figure can be increased to 40% if the pellets are washed. Alternatively the algae may be used as a food source for filter feeders such as young oysters and prawns.

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## STATEMENT

This work describes results and conclusions derived from 41 publications. The book, 'Seeds and their Uses' is not included.

Of these, 5 are under my sole authorship.

Responsibility for publication under joint authorship is allocated as follows:

Principal Author: papers numbered 1, 3, 6, 8, 11, 12, 14, 15, 16, 17, 19, 25, 26, 32, 36, 38, 39.

Major contributor: papers numbered 5, 9, 10, 13, 18, 20, 21, 22, 23, 24, 27, 28, 29, 31, 34, 35, 37, 40, 41.

The following papers include work which formed part of theses successfully submitted for the degree of Doctor of Philosophy by E.D. Baxter, 5, 9, 10, 13; A.R. Nutbeam, 18, 27; J.M. Williams, 20, 24. Papers numbered 38 and 39 include work which formed part of my own thesis submitted successfully for the degree of Doctor of Philosophy at the University of London (Imperial College). Paper number 41 includes work which was part of a thesis successfully submitted for the B.Sc. degree in Agriculture by N. Gibbs. The work described in papers numbered 5, 9, 10, 13, 18, 20, 23, 24, 27, 28, 29, 31, 34, 35, 37 was supported by grants made to me from the Agricultural Research Council, London.

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GROUP 1

Cereal Grain Development

## $\alpha$ -AMYLASE ACTIVITY IN THE DEVELOPING BARLEY GRAIN AND ITS DEPENDENCE ON GIBBERELIC ACID

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**Abstract**— $\alpha$ -Amylase activity was assayed in whole grains of barley from 3 days after anthesis to maturity and in starchy endosperm and aleurone from 16 days after anthesis to maturity. In all fractions there was maximal activity between 20 and 30 days after anthesis. The formation of  $\alpha$ -amylase was inhibited by application of the gibberellic acid synthesis inhibitor chlorocholine chloride (CCC) at a concentration of  $5 \times 10^{-4}$  M. The inhibition caused by CCC could be prevented by a subsequent treatment with gibberellic acid.

### INTRODUCTION

FEW, if any, reports exist of  $\alpha$ -amylase in the developing barley grain. May and Buttrose<sup>1</sup> measured the activity of a maltose producing enzyme system using starch as substrate in developing barley. Activity rose to a maximum around 30 days after anthesis. It was suggested that  $\beta$ -amylase was the major enzyme involved but no attempt was made to differentiate between  $\alpha$ - and  $\beta$ -amylase activity.

The presence of various hydrolytic enzymes other than  $\alpha$ -amylase has been demonstrated<sup>2</sup> in the developing grain of *Bromus ramosus*, a cereal grain similar to that of barley. While  $\alpha$ -amylase was apparently absent from all stages of the developing grain, both endo  $\beta$ -glucanase and pentosanase showed a rise and fall of activity with increasing maturity. The presence of  $\beta$ -amylase was also verified.

The present paper demonstrates the occurrence of  $\alpha$ -amylase in developing barley. The activity of this enzyme in extracts of whole grain, aleurone and endosperm, assayed from 3 days after anthesis to maturity, rose to maximum values between 20–30 days after anthesis. The application of CCC immediately after anthesis inhibited the formation of  $\alpha$ -amylase in the developing grain. The activity was normal if the application of CCC was followed by treatment with gibberellic acid.

### RESULTS

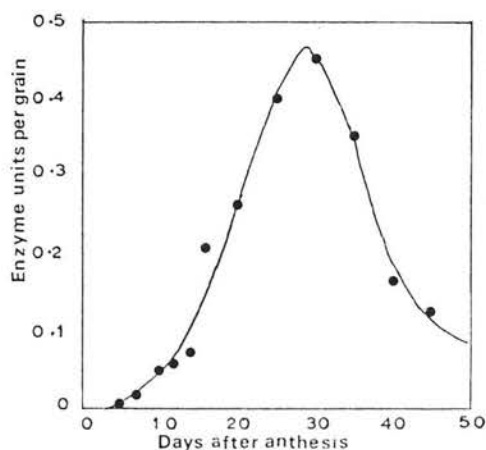
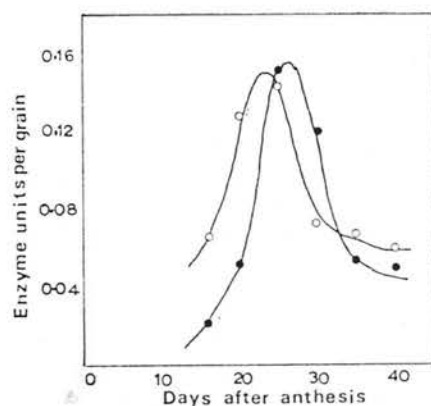
#### *Time Course of $\alpha$ -Amylase Activity in Extracts of Whole Grain, Endosperm and Aleurone*

Figure 1 shows the variation in extractable  $\alpha$ -amylase activity with age of whole grain. Activity rose slowly at first and then increased rapidly to a maximum value of 0.45 E.U./grain. At no time was there zero activity.

$\alpha$ -Amylase activity in both aleurone and endosperm extracts shows maximum values between 20–30 days after anthesis (Fig. 2). The maximum value for the aleurone layer is not significantly different from that of endosperm; it does, however, occur slightly later in the

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<sup>2</sup> C. S. JOHNSTON, Ph.D. thesis, University of Edinburgh (1965).

FIG. 1. TIME COURSE OF  $\alpha$ -AMYLASE ACTIVITY IN WHOLE GRAIN.FIG. 2. TIME COURSE OF  $\alpha$ -AMYLASE ACTIVITY IN STARCHY ENDOSPERM AND ALEURONE.

● Aleurone layer    ○ Starchy Endosperm.

time scale. When the  $\alpha$ -amylase of the embryo (due to contaminating aleurone) is taken into account, the sum of the activities of the three fractions agrees substantially with the value for the whole grain of the same age.

#### *Inhibition of $\alpha$ -Amylase Activity by CCC*

At 1 day after anthesis all the grains in one row of the ear (row A) were treated with  $5 \times 10^{-4}$  M CCC. The solution was applied with a fine paint brush. The other row (row B) was similarly treated with distilled water.  $\alpha$ -Amylase activity was then assayed in both rows 14 days after anthesis (Table 1). While no  $\alpha$ -amylase activity was apparent in row A, the activity of the control enzyme—glyoxalate NADP reductase—in both rows was not significantly different. The  $\alpha$ -amylase activity of row B was not significantly different from that of whole grains from untreated ears of the same age (see Fig. 1). This indicates that under the conditions used translocation of CCC from one row to the other was negligible and that at least some other enzymes are not affected by the inhibitor. It has also been shown that the presence of  $5 \times 10^{-4}$  M CCC in the enzyme system used does not inhibit enzyme activity. The results then show that CCC can inhibit  $\alpha$ -amylase formation in the developing barley grain.

TABLE 1. INHIBITION OF  $\alpha$ -AMYLASE FORMATION BY CCC

	Treatment	$\alpha$ -Amylase (E.U. units/grain)	Glyoxalate NADP reductase (absorptivity units/min/grain)
Row A	$5 \times 10^{-4}$ M CCC	0.0	$0.00062 \pm 0.00008$
Row B	H <sub>2</sub> O	0.086	$0.00056 \pm 0.00008$

If the CCC is applied later than 1 or 2 days after anthesis, inhibition of  $\alpha$ -amylase activity is not complete. Table 2 shows the variation in  $\alpha$ -amylase activity with date of application of CCC. Again enzyme activity was assayed in both rows 14 days after anthesis. The  $\alpha$ -amylase activity of row A, while still inhibited relative to row B, increases with increase in time of application of CCC after anthesis. Glyoxalate reductase activity in both row was at all times constant.

TABLE 2. VARIATION IN THE DEGREE OF INHIBITION OF  $\alpha$ -AMYLASE WITH DATE OF APPLICATION OF CCC

No. of days after anthesis when CCC applied	$\alpha$ -Amylase activity (E.U./grain)	
	Row A (+ $5 \times 10^{-4}$ M CCC)	Row B (H <sub>2</sub> O)
1	0.0	0.07
3	0.0103	0.07
4	0.013	0.06
7	0.035	0.10

#### *Restoration of $\alpha$ -Amylase Formation in the Presence of CCC by Gibberellic Acid*

In this experiment two ears (1 and 2) of the same age were selected. Two days after anthesis one row (row A) of each was treated with  $5 \times 10^{-4}$  M CCC and the other row (row B) with distilled water. Seven days after anthesis, row A of ear 1 was treated with  $5 \times 10^{-4}$  M gibberellic acid and the remaining rows with distilled water.  $\alpha$ -Amylase was assayed in each fraction 14 days after anthesis (Exp. 1). In a second experiment (Exp. 2)  $\alpha$ -amylase was assayed in each fraction 40 days after anthesis (Table 3). A separate experiment showed that the presence of  $5 \times 10^{-4}$  M gibberellic acid in the assay system used did not affect enzyme activity.

Table 4 shows the results obtained in a further experiment in which one ear only was used. In this case  $5 \times 10^{-4}$  M CCC was painted on row A, 2 days after anthesis. At 4 days the top six grains of row A were treated with  $5 \times 10^{-4}$  M gibberellic acid and the lower six of row A with distilled water. The inhibition of  $\alpha$ -amylase formation by CCC was reversed by gibberellic acid.

## DISCUSSION

The results in Figs. 1 and 2 show that  $\alpha$ -amylase activity in all grain extracts rose to a maximum value and decreased thereafter. Furthermore, the maximum value of  $\alpha$ -amylase activity in the aleurone layer occurs later in the time scale than that for starchy endosperm (Fig. 2). This suggests that in the developing grain some  $\alpha$ -amylase is synthesized in the

TABLE 3. REVERSAL OF THE INHIBITION OF  $\alpha$ -AMYLASE FORMATION BY GIBBERELIC ACID IN GRAINS TREATED WITH CCC USING TWO EARS OF THE SAME AGE

Experiment 1			
Treatment			$\alpha$ -Amylase (E.U./grain)
Ear 1	Row A	CCC + gibberellic acid ( $5 \times 10^{-4}$ M)	0.086
	Row B	H <sub>2</sub> O	0.086
Ear 2	Row A	CCC ( $5 \times 10^{-4}$ M)	0.00
	Row B	H <sub>2</sub> O	0.104
Experiment 2			
Treatment			$\alpha$ -Amylase (E.U./grain)
Ear 1	Row A	CCC + gibberellic acid ( $5 \times 10^{-4}$ M)	0.15
	Row B	H <sub>2</sub> O	0.11
Ear 2	Row A	CCC	0.04
	Row B	H <sub>2</sub> O	0.10

TABLE 4. RESTORATION OF  $\alpha$ -AMYLASE FORMATION BY GIBBERELIC ACID IN GRAINS TREATED WITH CCC USING ONE EAR ONLY

Treatment		$\alpha$ -Amylase (E.U./grain)
Row A (top six grains)	CCC + gibberellic acid ( $5 \times 10^{-4}$ M)	0.095
Row A (bottom six grains)	CCC ( $5 \times 10^{-4}$ M)	0.024
Row B	H <sub>2</sub> O	0.095

aleurone layer. The results are in agreement with the fact that the aleurone layer retains active anabolic systems after these have disappeared from the starchy endosperm.

The results show that the formation of  $\alpha$ -amylase in developing barley is inhibited by application of CCC (Table 1) and the amount of inhibition becomes less the later the date of application (Table 2). CCC is a gibberellin synthesis inhibitor in *Fusarium moniliforme*<sup>3</sup> (syn. *Gibberella fujikuroi*) and has been shown to inhibit the formation of a gibberellin like substance in barley embryos cultured on mevalonic acid<sup>4</sup> and in intact barley.<sup>5</sup> Furthermore, gibberellic acid synthesized in the embryo, has been implicated<sup>6,7</sup> in  $\alpha$ -amylase release by the aleurone layer into the starchy endosperm during barley germination. It may be, therefore, that  $\alpha$ -amylase synthesis in the developing seed is controlled by gibberellic acid too. Thus  $\alpha$ -amylase activity drops when synthesis of the hormone is prevented by CCC. That there are large amounts (3  $\mu$ g gibberellic acid/kg fresh weight) in the immature barley grain has been shown<sup>8</sup> by Jones *et al.* In addition, the results in Tables 3 and 4 show that gibberellic acid has a direct effect on  $\alpha$ -amylase synthesis in immature barley. The inhibition of  $\alpha$ -amylase formation by CCC was reversed by gibberellic acid and the enzyme activity restored to its normal level.

<sup>3</sup> H. NINNEMANN, J. A. D. ZEEVAART, H. KENDE and A. LANG, *Planta* **61**, 229 (1964).

<sup>4</sup> H. YOMO and H. IINUMA, *Planta* **71**, 113 (1966).

<sup>5</sup> D. E. BRIGGS, *Phytochem.* **7**, 539 (1968).

<sup>6</sup> H. YOMO, *Hakko Kyokaishi* **18**, 603 (1960).

<sup>7</sup> L. G. PALEG, *Plant Physiol.* **35**, 293 (1960).

<sup>8</sup> D. F. JONES, J. MACMILLAN and M. RADLEY, *Phytochem.* **2**, 307 (1963).

It appears likely, therefore, that the mechanism governing  $\alpha$ -amylase synthesis in the maturing grain is similar to that in germinating grain and is controlled by gibberellic acid. If the  $\alpha$ -amylase can be shown to be synthesized *de novo* in the developing grain, then this system might prove more convenient to use than the aleurone of mature grain in studies of the control of  $\alpha$ -amylase synthesis by gibberellic acid. Enzyme extraction is easier in younger tissue and pure preparations of endosperm and aleurone are not difficult to obtain.

## EXPERIMENTAL

### *Plant Material*

A two-row barley, *Hordeum distichum* (L.) Lam. var. Maris Baldric was used. The conditions of growth and the method used to determine the date of anthesis were as described by Merritt and Walker.<sup>9</sup> While normally used fresh from the growing ear, the grain could be stored for periods of up to two months at  $-12^{\circ}$  without loss of enzymatic activity.

### *Extraction of Enzyme*

Whole grain was dehusked by hand before use, leaving the aleurone as the outside layer. An aleurone fraction was prepared by removing the layer by hand and freeing it from endosperm as far as possible. However, some contamination of this fraction with endosperm in the older grain was inevitable. The endosperm fraction was that remaining after removal of embryo and aleurone. The material was homogenized in M NaCl (glass hand homogenizer) and centrifuged for 5 min in an MSE bench centrifuge at speed 10. The supernatant was used as the source of enzyme. In young material (3–10 days after anthesis) the concentration used was around 15 grains/ml M NaCl. Both  $\alpha$ -amylase and glyoxalate reductase were fully extracted in a 10 min period following homogenization. Older material (i.e. more than 15 days after anthesis), including endosperm and aleurone fractions, was used at a concentration of around 10 grains/3 ml M NaCl and a period of at least 1 hr following homogenization was required for complete extraction.

### *Enzyme Assays*

$\alpha$ -Amylase was assayed by the Iodine-Dextrin colour method of Briggs<sup>10</sup> but using his improved standard graph technique.<sup>11</sup> The activity was expressed in enzyme units (E.U.) per grain, where one enzyme unit is defined as  $100/t$ , where  $t$  is the time in min taken for the iodine-dextrin colour to fall to half its initial zero-time value. Glyoxalate NADP reductase was assayed by the method of Zelitch and Gotto<sup>12</sup> and the activity expressed in absorptivity units/min/grain. For any given fraction it was found that the standard deviation of the mean values quoted did not vary significantly. Thus the standard deviations could be averaged to give a more accurate assessment of the variability of the results. For aleurone, the average standard deviation was  $\pm 0.016$ , for endosperm  $\pm 0.004$ , for the whole grain 0.03 and for CCC-inhibited grain  $\pm 0.004$ .

*Acknowledgements*—The author thanks her husband Dr. J. H. Duffus for much useful advice and criticism and Mrs. Mary MacLean for expert technical assistance.

<sup>9</sup> N. R. MERRITT and J. T. WALKER, *J. Inst. Brewing*, in press (1969).

<sup>10</sup> D. E. BRIGGS, *J. Inst. Brewing* **67**, 427 (1961).

<sup>11</sup> D. E. BRIGGS, *J. Inst. Brewing* **73**, 361 (1967).

<sup>12</sup> I. ZELITCH and A. M. GOTTO, *Biochem. J.* **84**, 541 (1962).

## ENZYMATIC CHANGES AND AMYLOPLAST DEVELOPMENT IN THE MATURING BARLEY GRAIN

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**Abstract**—The changes in activity of various enzymes during maturation of the barley grain were recorded. Enzymes assayed were glyceraldehyde-3-phosphate:NAD and :NADP oxidoreductases, malate:NAD and :NADP oxidoreductases, glyoxalate:NAD and :NADP oxidoreductases, acid phosphatase, fumarase, catalase and peroxidase. The results were correlated with structural studies of amyloplast development.

### INTRODUCTION

LITTLE is known of the biochemical changes which take place in the developing cereal grain. The gross changes in the major storage materials such as protein, lipid and carbohydrates have been described by Jennings and Morton.<sup>1</sup> The ultrastructure of the developing wheat<sup>2</sup> and barley<sup>3</sup> endosperm has been described in detail by Buttrose. Marrè<sup>4</sup> has reviewed the maturation process in the castor bean seed and interpreted the course of differentiation at a molecular level by discussing the changes in enzyme activity which take place during seed development. It was found that most of the enzymes involved in basic metabolism (e.g. malate dehydrogenase, fructose-1,6-diphosphatase, aldolase, etc.) followed a similar pattern of activity throughout maturation. Thus activity increased rapidly during seed development to a maximum when maximum fresh weight was first reached, and thereafter decreased to the low levels characteristic of the mature seed. Maturation is characterized by the conversion of sugar to lipid and by the synthesis of reserve protein and phytin and these changes were correlated with the changes in enzyme activity.

The main emphasis in this paper is on the changes in enzyme activities taking place during maturation of barley seeds and correlation of the results with the development of amyloplasts.

### RESULTS

*Peroxidase and Catalase (Donor: H<sub>2</sub>O<sub>2</sub> Oxidoreductase EC 1.11.1.7 and H<sub>2</sub>O<sub>2</sub>:H<sub>2</sub>O<sub>2</sub> Oxidoreductase EC 1.11.1.6)*

Figure 1 shows the variation in extractable peroxidase and catalase with age of whole grain. Activity of both enzymes remains low throughout the first 10–12 days of maturation after which it rises sharply to a maximum value around 15–20 days after anthesis. Activity then falls off rapidly with increasing maturity of the grain.

Edinburgh School of Agriculture, Miscellaneous Publication No. 457.

<sup>1</sup> A. C. JENNINGS and R. K. MORTON, *Australian J. Biol. Sci.* **16**, 318, 332 (1963).

<sup>2</sup> M. S. BUTTROSE, *Australian J. Biol. Sci.* **16**, 305 (1963).

<sup>3</sup> M. S. BUTTROSE, *J. Ultrastruct. Res.* **4**, 231 (1960).

<sup>4</sup> E. MARRÈ, in *Current Topics in Development Biology* (edited by A. A. MOSCONA and A. MONROY), Vol. 2, p. 76, Academic Press, New York (1967).

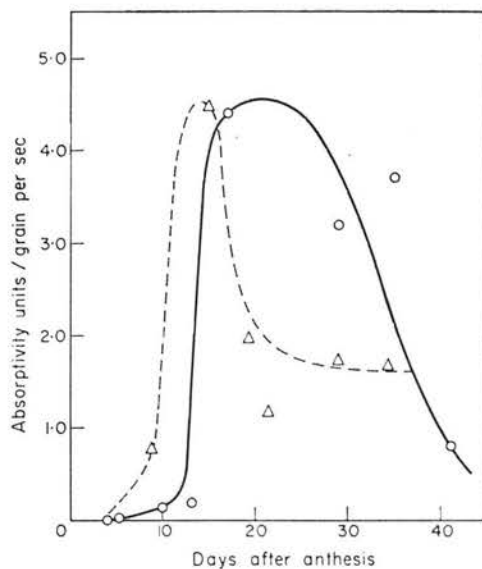


FIG. 1. CHANGES IN PEROXIDASE (○—○) AND CATALASE (Δ---Δ: VALUES  $\times 10^3$ ) ACTIVITY DURING MATURATION OF WHOLE GRAIN.

*Glyceraldehyde-3-phosphate NAD and NADP Dehydrogenases* (D-Glyceraldehyde-3 Phosphate:NAD Oxidoreductase (Phosphorylating), EC 1.2.1.12 and D-Glyceraldehyde-3-phosphate:NADP Oxidoreductase (Phosphorylating), EC 1.2.1.13)

The activity of the NAD enzyme rises steadily throughout the early stages of maturation, increasing rapidly to a maximum about 20 days after anthesis and falling off rapidly there-

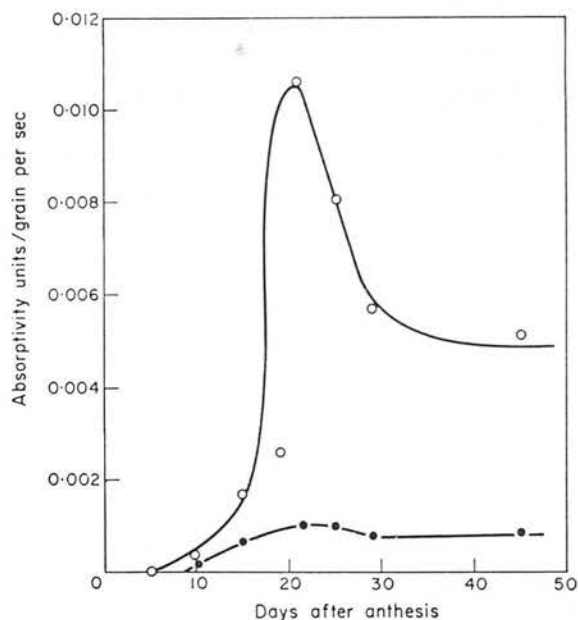


FIG. 2. CHANGES IN GLYCERALDEHYDE-3-PHOSPHATE NAD (○—○) AND NADP (●—●) DEHYDROGENASES ACTIVITY DURING MATURATION OF WHOLE GRAIN.



after (Fig. 2). The NADP enzyme has a much lower activity and shows little variation throughout the maturation period.

*Malate NAD and NADP Dehydrogenases* (L-Malate:NAD Oxidoreductase, EC 1.1.1.37 and L-Malate:NADP Oxidoreductase)

Results are similar to those for the glyceraldehyde-3-phosphate dehydrogenases except that the peaks of activity are rather later in the time scale (Fig. 3). Again the NADP enzyme is present but the activity is very low.

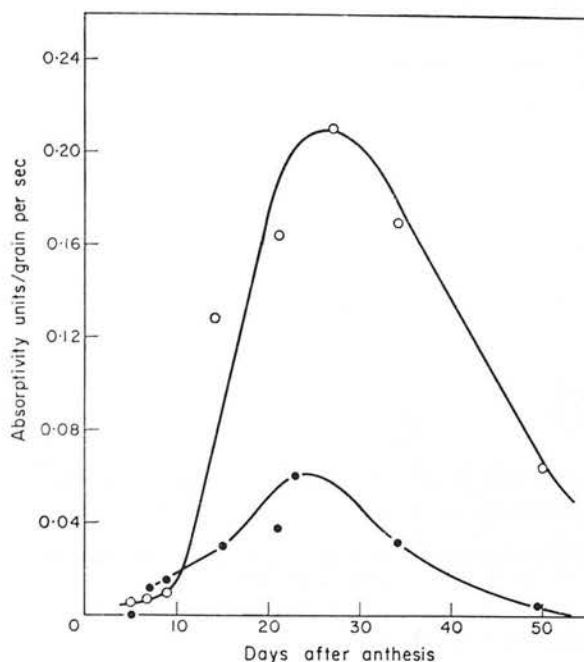


FIG. 3. CHANGES IN MALATE NAD (○—○) AND NADP (●—●: VALUES  $\times 10^2$ ) DEHYDROGENASES ACTIVITY DURING MATURATION OF WHOLE GRAIN.

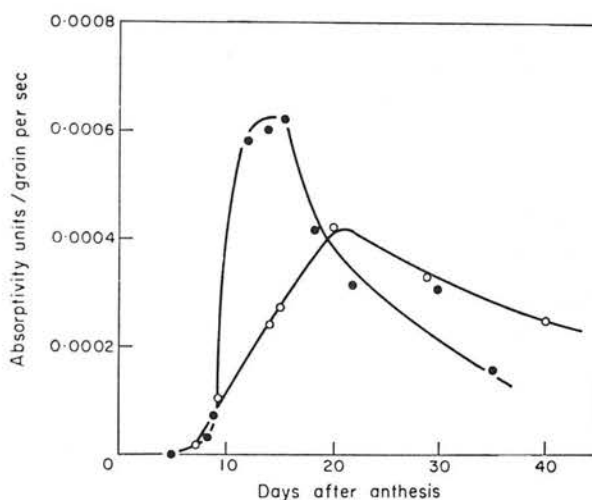


FIG. 4. CHANGES IN GLYOXALATE NAD (○—○) AND NADP (●—●) DEHYDROGENASES ACTIVITY.

*Glyoxalate NAD and NADP Reductases (Glycollate:NAD Oxidoreductase, EC 1.1.1.26 and Glycollate:NADP Oxidoreductase)*

Again, both NAD and NADP enzymes are present (Fig. 4), in this case with similar (and low) activities. The maximum for the NADP enzyme occurs slightly earlier in the maturation period and falls off more rapidly than that for the NAD enzyme.

*Acid Phosphatase (Orthophosphoric Monoester Phosphohydrolase EC 3.1.3.2)*

Activity rises steadily throughout maturation with a maximum value (Fig. 5) around 25–30 days after anthesis.

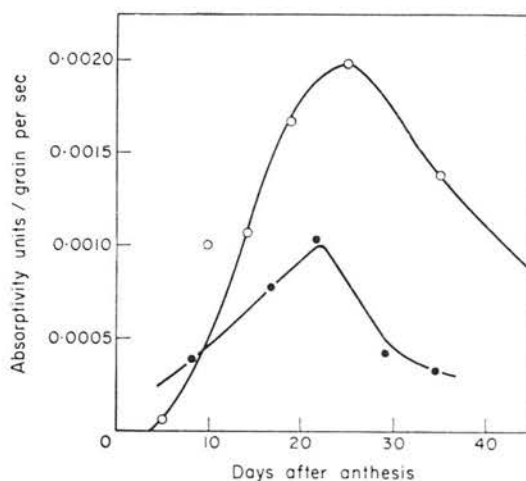


FIG. 5. CHANGES IN ACID PHOSPHATASE (○—○) AND FUMARASE ACTIVITY (●—●) DURING MATURATION OF WHOLE GRAIN.

*Fumarase (L-Malate Hydro-lyase, EC 4.2.1.2)*

Activity rises slowly to a maximum around 21 days after anthesis and decreases steadily throughout the later stages of maturation (Fig. 5).

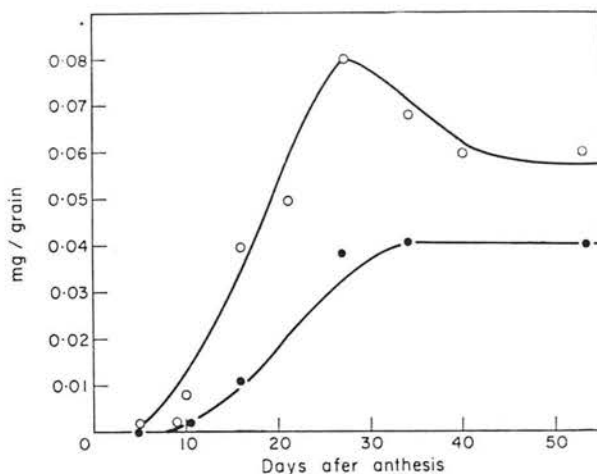


FIG. 6. CHANGE IN FRESH (○—○) AND DRY (●—●) WEIGHT DURING MATURATION OF WHOLE GRAIN.

### *Fresh and Dry Weights*

The results (Fig. 6) are very similar to those for developing whole wheat grain.<sup>1</sup> Dry weight reaches a plateau around 30 days after anthesis and fresh weight a maximum value around 25 days after anthesis.

### *Light Microscopy*

Starch-containing plastids could be seen in the barley grain from 5 days after anthesis. During the period 5–21 days after anthesis the starch granules grow rapidly to fill the plastid and any internal structures previously visible become obscured. The size of the starch granule reached a maximum around 21 days after anthesis. In addition to the large amyloplasts, a considerable number of smaller starch granules could be seen.

## DISCUSSION

The ultrastructure of the developing barley grain has been described by Buttrose.<sup>3</sup> The proplastids of the young endosperm, from which amyloplasts subsequently develop, were seen as early as 5 days after anthesis. Invaginations into the plastid stroma parallel to the inner membrane illustrate their similarity to the normal chloroplast. Initiation of starch granules in proplastids was observed to continue for about 14 days after anthesis.

Light microscopy was used in the present case to follow the gross detail of amyloplast development throughout grain maturation and the results agreed substantially with those described above. The smaller starch granules were also described by Buttrose<sup>3</sup> and are derived apparently from the amyloplast itself.

The period of decay of proplastid internal structure coincides with the rapid increase in activity of the two enzymes catalase and peroxidase. Perhaps both enzymes are products of this plastid disintegration. One consequence of increased peroxidase activity may be a marked decrease in indole acetic acid concentration.<sup>5</sup> That this may have an important effect on enzyme levels in the endosperm has been shown by MacLeod and Palmer.<sup>6</sup>

The development of plastids into starch-accumulating particles or amyloplasts is accompanied by a rapid increase in the enzymes of carbohydrate metabolism, glyceraldehyde-3-phosphate NAD and NADP dehydrogenases. Again, the maximum in activity coincides with the maximum size of the amyloplast. While both enzymes may be concerned with hexose synthesis the NADP enzyme has been assigned to a primarily photosynthetic role.<sup>7</sup> Smillie and Fuller<sup>8</sup> found it only in green plants and in oxygen evolving photosynthetic organisms. Although it does have a broadly similar pattern of activity over the period of maturation, this activity is much less than that for the NAD enzyme. The latter enzyme is thought to participate in the dark reactions of photosynthesis,<sup>8</sup> possibly by means of an NADPH transhydrogenase, and certainly about 30 per cent of the whole cell activity of spinach leaves has been localized in the chloroplast.<sup>9</sup>

Alternatively the NAD enzyme may be involved in glycolysis. The results agree well with those of Hageman and Arnon<sup>10</sup> for pea seeds and leaves who found that the developing seed had much less NADP glyceraldehyde-3-phosphate dehydrogenase than NAD enzyme. On the other hand the same enzymes in green leaves had almost equal activities.

<sup>5</sup> P. M. RAY, *Ann. Rev. Plant Physiol.* **9**, 81 (1958).

<sup>6</sup> A. M. MACLEOD and G. H. PALMER, *New Phytol.* **68**, 295 (1969).

<sup>7</sup> G. A. HUDOCK and R. C. FULLER, *Plant Physiol.* **40**, 1205 (1965).

<sup>8</sup> R. M. SMILLIE and R. C. FULLER, *Biochem. Biophys. Res. Commun.* **3**, 368 (1960).

<sup>9</sup> U. HEBER, N. G. PON and M. HEBER, *Plant Physiol.* **38**, 355 (1963).

<sup>10</sup> R. H. HAGEMAN and D. I. ARNON, *Arch. Biochem. Biophys.* **57**, 421 (1955).

Lipid is present in most of the tissues of the barley grain and it has been shown<sup>1</sup> that endosperm lipid content increases during the first 4 weeks of wheat grain maturation. It is probable that the malic dehydrogenase and glyoxalate reductase are concerned in pathways of lipid metabolism. Glyoxalate NADP reductase has been shown to be localized in leaf chloroplasts<sup>11</sup> and malate NADP dehydrogenase is probably present there also.<sup>12</sup> A scheme for acetyl CoA synthesis in plastids involving both these enzymes has been proposed by Stumpf *et al.*<sup>13</sup> Part, at least, of the NADH malic dehydrogenase and of the NADH glyoxalate reductase is present in plastids and therefore may be also associated with the amyloplast. The increase in the amount of lipid formed probably correlates with the increase in the number of intracellular membranes abundant in endosperm cells during maturation.<sup>3</sup>

That a reducing environment, conducive to lipid and, indeed, carbohydrate synthesis, may prevail in the maturing grain is borne out by the decrease in activity of the mitochondrial enzyme fumarase throughout the later stage of maturation. Certainly, in developing castor bean endosperm—a lipid synthesizing tissue—mitochondrial enzyme activity decreases from the onset of the constant fresh weight phase.<sup>4</sup> A supply of reduced nucleotides should then be available for both lipid and carbohydrate synthesis.

It will be noted that the peak of activity for NADPH glyoxalate reductase is somewhat earlier in the time scale than that for the NADH enzyme and corresponds roughly in time to the period of rapid increase in catalase and peroxidase activity. This may be correlated with a switch from purely plastid lipid synthesis to intracellular membrane synthesis.

The presence of various hydrolytic enzymes other than phosphatase, of which  $\alpha$ -amylase is one, has been demonstrated in the developing grain.<sup>14,15</sup> The activity of such enzymes increases rapidly during germination and contributes to the mobilization of storage material. Their significance to the developing grain is obscure. It may be that, like  $\alpha$ -amylase, phosphatase activity is controlled by gibberellic acid during the maturation process.

## EXPERIMENTAL

### *Plant Material*

The two-row barley, *Hordeum distichum* (L.) Lam. cv. Maris Baldric, was used. The conditions of growth and the method used to determine the date of anthesis were as described by Merritt and Walker.<sup>16</sup> Grain could be stored for periods of up to 2 months at  $-12^{\circ}$  without loss of enzymatic activity.

### *Extraction of Enzymes*

Enzymes were extracted using a glass homogenizer followed by brief centrifugation as previously described.<sup>15</sup> The extraction medium was the buffer used in the subsequent assay systems.

### *Enzyme Assays*

Enzymes were assayed using Unicam SP500 (single beam) and SP800 (double beam) spectrophotometers. Catalase,<sup>17</sup> peroxidase<sup>18</sup> (using *O*-dianisidine as hydrogen donor), acid phosphatase,<sup>19</sup> glyceraldehyde-3-

<sup>11</sup> C. M. THOMPSON and C. P. WHITTINGHAM, *Biochim. Biophys. Acta* **143**, 642 (1967).

<sup>12</sup> M. D. HATCH and C. R. SLACK, *Biochem. Biophys. Res. Commun.* **34**, 589 (1969).

<sup>13</sup> P. K. STUMPF, J. BROOKS, T. GALLIARD, J. C. HAWKE and R. SIMONI, in *Biochemistry of the Chloroplasts* (edited by T. W. GOODWIN), Vol. II, p. 213, Academic Press, New York (1967).

<sup>14</sup> C. S. JOHNSTON, Ph.D. Thesis, University of Edinburgh (1965).

<sup>15</sup> C. M. DUFFUS, *Phytochem.* **8**, 1205 (1969).

<sup>16</sup> N. R. MERRITT and J. T. WALKER, *J. Inst. Brewing* **75**, 156 (1969).

<sup>17</sup> H. LÜCK, in *Methods of Enzymatic Analysis* (edited by H. U. BERGMAYER), p. 885, Academic Press, New York (1965).

<sup>18</sup> H. LÜCK, in *Methods of Enzymatic Analysis* (edited by H. U. BERGMAYER), p. 895, Academic Press, New York (1965).

<sup>19</sup> K. LINHARDT and K. WALTER, in *Methods of Enzymatic Analysis* (edited by H. U. BERGMAYER), p. 779, Academic Press, New York (1965).

phosphate NAD and NADP dehydrogenases,<sup>10</sup> malate NAD and NADP dehydrogenases,<sup>20</sup> glyoxalate NAD and NADP reductases,<sup>21</sup> and fumarase<sup>22</sup> were assayed by standard techniques. Activities were expressed in absorptivity units per sec per grain.

#### *Light Microscopy*

Samples for examination were homogenized gently in M NaCl and filtered through two layers of muslin. The solutions were examined using a light microscope equipped with phase contrast (magnification  $\times 1000$ ). The preparations were stained for starch when necessary with  $I_2/KI$ .

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I also wish to thank Dr. Trevor Walker of the Scottish Plant Breeding Station, Pentlandsfield, East Lothian, for his kindness in supplying the plant material.

<sup>20</sup> A. KORNBERG and H. BEEVERS, *Biochim. Biophys. Acta* **26**, 531 (1957).

<sup>21</sup> I. ZELITCH and A. M. GOTTO, *Biochem. J.* **84**, 541 (1962).

<sup>22</sup> W. S. PIERPOINT, *Biochem. J.* **75**, 511 (1960).

## SHORT COMMUNICATION

# STARCH SYNTHETASE IN DEVELOPING BARLEY AMYLOPLASTS

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**Abstract**—The activity of starch synthetase in amyloplasts from barley endosperm was measured. The relative effectiveness of UDPG and ADPG as glucosyl donors was determined, and correlated with amyloplast age. Only UDPG linked synthesis could be detected up to about 12 days after anthesis. After this time ADPG was the main glucosyl donor.

## INTRODUCTION

THE INVOLVEMENT of sugar nucleotides in starch synthesis was first reported by De Fekete *et al.*<sup>1</sup> Since then both UDP and ADP<sup>2, 3</sup> linked mechanisms have been observed in many plant tissues. There is some evidence that the rate of glucose transfer from ADPG may exceed that from UDPG.<sup>3</sup> This paper describes an investigation into the relative importance in the developing amyloplasts of UDP glucose  $\alpha$ 1-4 glucan  $\alpha$ 4 glucosyl transferase (E.C. 2.4.111 starch synthetase) and the corresponding activity using ADPG as glucosyl donor.

## RESULTS

Starch was visible in the plastids from 2 to 3 days after anthesis, although starch synthetase activity was detected only from 6 days after anthesis. Plastid size increased until 25 days. From 21 days onward small plastids could be seen inside the larger ones.<sup>4</sup> No starch was detected in the soluble fraction.

Both UDPG and ADPG linked starch synthetase increased in activity with plastid size (Fig. 1). However, the relative activity of the UDPG enzyme decreased with increasing plastid size (Fig. 2) whilst the ADPG enzyme became increasingly important. When starch was added to the assay system there was an increase in activity in the plastids. The percentage increase was greatest with very young plastids (about 6-7 days after anthesis) indicating that at this stage of development primer concentration is limiting.

The starch synthetase activity of the soluble fraction at first appeared to be greater than that of plastids of the same age. The activity of the soluble fraction of 14 day grains exceeded that of plastids of the same age by a factor of  $10^3$  (using UDPG) or  $10^2$  (using ADPG), when assayed by UDP (or ADP) production. There was, however, no stimulation of activity when starch was added as a primer, although the original soluble fraction did not contain any

<sup>1</sup> M. A. R. De FEKETE, L. F. LOLOIR and C. E. CARDINI, *Nature* **187**, 918 (1960).

<sup>2</sup> L. F. LOLOIR, M. A. R. De FEKETE, and C. E. CARDINI, *J. Biol. Chem.* **236**, 636 (1961).

<sup>3</sup> E. RECONDO and L. F. LOLOIR, *Biochem. Biophys. Res. Commun.* **6**, 85 (1961).

<sup>4</sup> M. S. BUTTROSE, *J. Ultrastruct. Res.* **4**, 231 (1960).

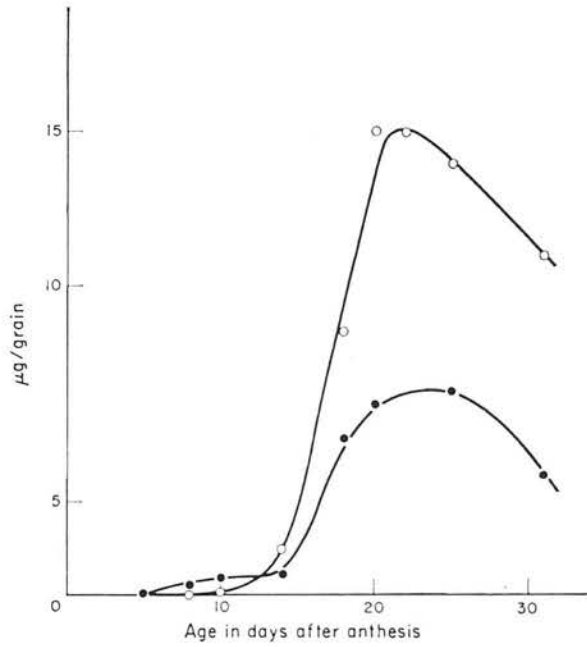


FIG. 1. LEVELS OF NUCLEOSIDE PYROPHOSPHATES SYNTHESIZED BY PLASTIDS DURING 2 hr INCUBATION IN  $\mu\text{gm}/\text{grain}$ .

ADP and UDP values corrected for endogenous levels (see text) ● UDP; ○ ADP.

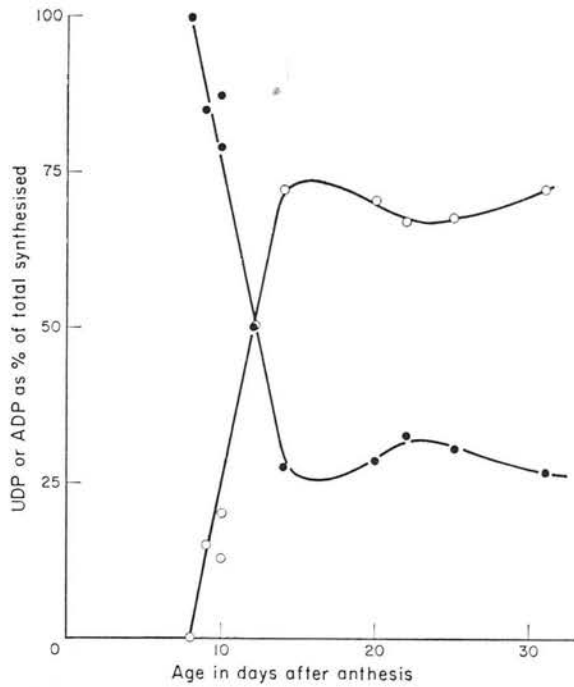


FIG. 2. AMOUNT OF UDP (OR ADP) SYNTHESIZED BY PLASTIDS EXPRESSED AS A PERCENTAGE OF TOTAL UDP + ADP SYNTHESIZED.

● UDP; ○ ADP.

detectable starch. Enzyme fractions were therefore incubated with UDP ( $^{14}\text{C}$ ) G and starch as a carrier. Incorporation of ( $^{14}\text{C}$ ) glucose into an insoluble product associated with the starch pellet occurred only with the plastid fraction. This suggests that the high concentrations of UDP and ADP detected in the previous assays of the soluble fraction are formed by the breakdown of the UDPG and ADPG added without the glucosyl moiety being incorporated into a high molecular weight polymer. These high levels of UDP and ADP may be due to the activity of sucrose synthetase, an enzyme which is generally reversible,<sup>5</sup> although synthesis of sucrose may be somewhat slower than the reverse reaction at physiological pH.<sup>6</sup> This enzyme is highly reactive in the soluble fraction of barley grain homogenates. The specific activity (in nmoles sucrose/min/grain) increased from 1.4 at 7 days after anthesis to a peak of 624 at 28 days, afterwards levelling off to a value of around 500 in the mature grain. Similar, or slightly lower, levels of activity in the direction of sucrose synthesis, could account for all the UDP and ADP produced by the soluble fraction during the assay for starch synthetase by the pyruvate kinase method. No sucrose synthetase activity could be detected in the plastids.

### DISCUSSION

These results agree with those of previous workers<sup>3, 7, 8</sup> which suggest that proliferation of starch in developing seeds is mainly brought about by ADPG linked starch synthetase associated with the amyloplasts. Starch is, however, visible in the plastids before starch synthetase activity can be detected. Also, this enzyme has been shown to require an oligosaccharide primer.<sup>2</sup> These observations suggest that a different enzyme is involved in the early synthesis of starch from low molecular weight primers. Tsai and Nelson<sup>9</sup> have isolated four phosphorylase isoenzymes from maize, three of which are present in the developing endosperm and one in the embryo. Their work suggests that two of the endosperm isoenzymes may be capable of synthesizing starch without the addition of primer. Slabnik and Frydman<sup>10</sup> have detected a similar phosphorylase enzyme in potatoes. Classical phosphorylase activity can be detected in the soluble fraction of young barley endosperm, using soluble starch as a primer. This activity increased from 195 ng starch produced/grain/min at 12 days after anthesis to 334 ng at 19 days. So far attempts to obtain synthesis of starch from glucose-1-phosphate by phosphorylase without added primer have been unsuccessful. However, there is some evidence<sup>9</sup> that an inhibitor of certain phosphorylase isoenzymes may be present in crude cereal grain homogenates. Bird<sup>11</sup> has shown that in destarched chloroplasts supplied with glucose-1-phosphate, phosphorylase will bring about the synthesis of an insoluble glucose polymer which is sufficient to prime the starch synthetase reaction. In young barley endosperm, such a primer would be slowly converted into starch by UDPG linked starch synthetase. By 12–15 days after anthesis, when ADPG linked starch synthetase has become more active, the starch content of the plastids increases rapidly and starch is no longer limiting. At this later stage UDP and UDPG may be involved in the utilisation, via sucrose synthetase, of the sucrose supplied to the endosperm by the leaves.<sup>5, 6</sup>

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<sup>6</sup> R. PRESSEY, *Plant Physiol.* **44**, 759 (1969).

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<sup>8</sup> L. C. BAUN, E. P. PALMIANO, C. M. PEREZ and B. JULIANO, *Plant Physiol.* **46**, 429 (1970).

<sup>9</sup> C. Y. TSAI, O. E. NELSON, *Plant Physiol.* **44**, 159 (1969).

<sup>10</sup> E. SLABNIK and R. FRYDMAN, *Biochem. Biophys. Res. Commun.* **38**, 709 (1969).

<sup>11</sup> I. F. BIRD, Ph.D. Thesis, University of London, 1969.



## EXPERIMENTAL

*Plant material.* Two row barley plants (*Hordeum distichium* (L.). Lam. v. Maris Baldric) were used. Conditions of growth and methods used to determine the date of anthesis were as described by Merritt and Walker.<sup>12</sup> Grains could be stored at  $-15^{\circ}$  for 3 months without loss of activity.

*Preparation of amyloplasts.* Endosperms were separated from embryo and aleurone by hand, homogenised in an all-glass Potter type homogenizer in 2 vol. of  $H_2O$  and filtered through double muslin to remove cell debris. Amyloplasts were separated from the soluble components by centrifugation for 10 min at  $4^{\circ}$  and 2500 g. They were resuspended in  $H_2O$ .

*Enzyme assays.* Starch synthetase was assayed by the method of Leloir,<sup>2</sup> but in a total volume of 0.4 ml using 0.1 ml of the suspended amyloplasts as the source of enzyme. The supernatant solution from the centrifugation, containing the soluble components of the endosperm, was also assayed for synthetic activity. All assays were corrected for endogenous nucleotides using controls incubated without UDPG or ADPG.

ADP and UDP were measured by the pyruvate kinase method<sup>13</sup> and compared with standards run simultaneously. Assays carried out without amyloplasts showed that neither UDPG nor ADPG dissociated under the conditions of the assays. Starch synthetase assays were also carried out using  $^{14}C$  labelled UDPG (see Leloir).<sup>2</sup> UDP-( $^{14}C$ ) G (ammonium salt, 237 mc/mM) was obtained from the Radiochemical Centre, Amersham. The soluble fraction was assayed for phosphorylase by the method of Slabnik and Frydman.<sup>10</sup> Sucrose synthetase was assayed by the method of Avigad and Milner.<sup>14</sup>

*Light microscopy.* Each amyloplast preparation was examined using a light microscope equipped with phase contrast lenses (magnification  $\times 500$ ). An age approximating to the number of days after anthesis was assigned to each preparation according to the average plastid size. The presence of starch was detected by staining with a 2% KI-0.2%  $I_2$  solution.

*Acknowledgements*—The authors wish to thank the Scottish Plant Breeding Station, Pentlandsfield, East Lothian, for supplying the plant material. The microscope, a Vickers M15C, was a Royal Society Grant. The work was supported by a grant from the Agricultural Research Council.

<sup>12</sup> N. R. MERRITT and J. T. WALKER, *J. Inst. Brewing* **75**, 156 (1969).

<sup>13</sup> L. F. LOLOIR and S. H. GOLDBERG, *Methods in Enzymology*, Vol. V, p. 145, Academic Press, London (1962).

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SEPARATUM

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## Characterization of Nuclei from Immature Barley Endosperm

by C. M. Duffus, Edinburgh (Scotland, U.K.)

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## Characterization of Nuclei from Immature Barley Endosperm

The ultrastructure of the developing barley endosperm has been described by BUTTROSE<sup>1</sup>. Little is known, however, of the biochemical events following anthesis and, in particular, of the role of the nucleus in these events.

Few convenient methods for the isolation of nuclei from higher plants have been reported, mainly because of the presence in the selected tissues of chloroplasts, amyloplasts and mechanically resistant cell walls. In the cereal grain, however, immediately after anthesis, rapid division of the triploid endosperm nucleus takes place without accompanying cell wall formation<sup>2</sup>. The nuclei are close packed with only a few undifferentiated spherical bodies present. Thus a method could be easily devised for the isolation of relatively uncontaminated and intact nuclei. This paper then describes some of their properties in terms of chemical composition, metabolic activity and histone content.

**Materials and methods.** The 2 row barley, *Hordeum distichum* (L.) Lam. CV. MARIS BALDRIC, was used and was grown either in quantity on the University farms or in the greenhouse under lights.

100 grains, never older than 3 days after anthesis were individually squashed in an ice-cold agate mortar in 2 ml of *M* sorbitol containing 30% glycerol and 0.001 *M* Ca<sup>2+</sup>. The mixture was filtered through 2 layers of muslin on to 2.0 ml of 1.8 *M* sorbitol, containing 30% glycerol and 0.001 *M* Ca<sup>2+</sup> in a 5 ml polypropylene centrifuge tube. Thus 2 layers were formed. A glass rod was passed once through the interface to give a slight mixing of the layers. This was centrifuged for 20 minutes at 200 × *g* and the nuclei accumulated in the lower layer. The top layer and interface were discarded. The lower layer was then transferred to another 5 ml centrifuge tube and centrifuged at 200 × *g* for 2 h. The pellet was further compacted by accelerating to 1,000 × *g* for 1 min at the end of the 2 h. This pellet was used without further treatment in the experiments to be described.

The purification was monitored routinely with a Vickers Patholux microscope using phase contrast lenses and a magnification of 1,000 diameters. A Leitz microscope was used to take phase contrast photomicrographs at a magnification of 700 diameters.

DNA, RNA and protein were determined in the nuclear pellet as described by ROZIJN and TONINO<sup>3</sup>. RNA polymerase was assayed as described by WEISS<sup>4</sup> using boiled nuclei as a control. The pH of assay was 7.8. NAD pyrophosphorylase was assayed by the method of GINSBURG-TIETZ et al.<sup>5</sup> and the NAD synthesized measured by the method of KLINGENBERG<sup>6</sup>. Fumarase was assayed by the method of PIERPOINT<sup>7</sup> and succinoxidase spectrophotometrically as described by VEEGER et al.<sup>8</sup>.

Histones were extracted from the nuclear pellet by a modification of the method of MURRAY et al.<sup>9</sup>. The nuclei were extracted with 3.0 ml of a KCl-HCl buffer ionic strength, 0.1, pH 2.8, and the suspension left at 4°C for 20 min before centrifuging at 5,000 × *g* for 10 min. The supernatant was discarded and the pellet washed once with 3.0 ml of the same buffer. The washed pellet was then

<sup>1</sup> M. S. BUTTROSE, *J. Ultrastruct. Res.* **4**, 231 (1960).

<sup>2</sup> C. YAMPOLSKY, *Wallerstein Labs Commun.* **20**, 343 (1957).

<sup>3</sup> Th. H. ROZIJN and G. J. M. TONINO, *Biochim. biophys. Acta* **91**, 105 (1964).

<sup>4</sup> S. B. WEISS, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN; Academic Press, New York 1968), vol. 12B, p. 557.

<sup>5</sup> Y. GINSBURG-TIETZ, E. KAUFMANN and A. TRAUB, *Biochim. biophys. Acta* **134**, 211 (1967).

<sup>6</sup> M. KLINGENBERG, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMAYER; Academic Press, New York 1965), p. 528.

<sup>7</sup> W. S. PIERPOINT, *Biochem. J.* **75**, 511 (1960).

<sup>8</sup> C. VEEGER, D. V. DERVARTANIAN and W. P. ZEYLEMAKER, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN; Academic Press, New York 1969), vol. 13, p. 81.

<sup>9</sup> K. MURRAY, G. VIDALI and J. M. NEELIN, *Biochem. J.* **107**, 207 (1968).

extracted with 3.0 ml 0.25 M HCl and the suspension centrifuged at  $5,000 \times g$  for 10 min. The supernatant was removed with a Pasteur pipette and 9 volumes of acetone at  $-18^\circ\text{C}$  added to it. The mixture was left overnight at  $-18^\circ\text{C}$  and the resulting precipitate pelleted and washed 3 times with acetone before drying under vacuum.

Histones were extracted from whole cells by squashing 60 whole grains in 2 ml of HCl-KCl buffer pH 2.8, ionic strength 0.1, in an agate mortar. After 20 min at  $4^\circ\text{C}$  the mixture was spun at  $5,000 \times g$  for 20 min without filtering. The supernatant was discarded and the pellet washed with a further 3 ml of the same buffer. The pellet was then homogenized in a glass homogenizer with 3 ml 0.25 M HCl, left for 20 min at  $4^\circ\text{C}$ , and centrifuged at  $5,000 \times g$  for 20 min. The histones were precipitated from the supernatant as described above.

Polyacrylamide gel electrophoresis was carried out by the method of JOHNS<sup>10</sup> using the Shandon disc electrophoresis apparatus and Vokam power unit.

**Results and discussion.** The isolated nuclei are extremely fragile and rupture under even the slight mechanical pressure of a cover slip. Thus, since they are obtained as a concentrated suspension rather than as a pellet and applied to the slide in a larger than ideal volume, it is difficult to obtain a uniform focal plane for more than a few nuclei (Figure 1). The nuclei are large,  $3\text{--}5\ \mu\text{m}$  and very similar to those seen in fixed and stained sections of the original tissue. Examination of the nuclear preparations by phase contrast microscopy revealed little cytoplasmic contamination. The mitochondrial enzymes succinoxidase and fumarase, often used as an indication of cytoplasmic contamination, were not detected in the nuclear pellet. Thus the preparation satisfies the criteria for morphological appearance under phase contrast, nuclear enzyme activity and low cytoplasmic activity suggested by ROODYN<sup>11</sup>.

The nuclei are characterized by a relatively low DNA:RNA ratio compared to pea seedling nuclei<sup>12</sup> (Table I). In

whole grain sections fixed and stained with Feulgen, endosperm nuclei have a much lower colour intensity than those in the surrounding tissue. This is consistent with the low nuclear DNA concentration. Furthermore the endosperm nuclei are associated with a rapidly developing tissue and soon after the period of free nuclear division, cell walls are formed, amyloplasts quickly fill the cells and the nuclei disappear. Endosperm protein synthesis still continues in their absence<sup>13</sup>. Thus the nuclei, at the time of isolation must be actively synthesizing both ribosomal and messenger RNA and this might account for a high nuclear RNA content.

The isolated nuclei are metabolically active and have appreciable RNA polymerase and NAD pyrophosphorylase activity (Table II). That the RNA polymerase was DNA dependent was shown by a 5-fold decrease in activity following a 20 min preincubation of the nuclei with deoxyribonuclease. A significant decrease in activity was also observed by a 5 min post-incubation treatment with ribonuclease showing that the radioactive product was indeed RNA. The results for RNA polymerase and NAD pyrophosphorylase compare favourably with those for pea<sup>12</sup> and yeast<sup>14</sup> nuclei, respectively. Results with animal cell nuclei<sup>15</sup> have shown that RNA polymerase is firmly bound to DNA and that NAD pyrophosphorylase is associated with nuclear ribosomes isolated after disruption of the nuclear membrane<sup>16</sup>. An RNA polymerase in plant cell nuclei has been demonstrated by RHO and CHIPCHASE<sup>17</sup>.

The disc electrophoretic patterns of histones from endosperm nuclei (B) and intact endosperm cells (C) run simultaneously with a control of calf thymus histone are shown in Figure 2. The first 2 bands are impurities found at the origin and may be ignored. Only one histone component can be seen in the nuclear extract. This has a mobility between that of the first and second calf thymus histones. Whole cells have 3 additional components of which 2 are



Fig. 1. Phase contrast micrograph of barley endosperm nuclei isolated by the method described in the text.

Table I. Chemical composition of isolated nuclei

Tissue	DNA (%)	RNA (%)	Protein (%)
Nuclei	$7 \pm 2.9$ (5)	$16 \pm 5$ (5)	$77 \pm 4$ (5)
Whole endosperm cells	$1.0 \pm 0.5$ (3)	$24 \pm 8.5$ (3)	$75 \pm 7.6$ (3)
Pea seedling nuclei (BIRNSTIEL et al. <sup>12</sup> )	13	10	77

For each experiment the sum of DNA, RNA and protein is set at 100. The results are expressed as percentages of this sum, with standard deviations. The figures for whole endosperm cells and pea seedling nuclei are given for comparison. The number of experiments are given in parenthesis.

<sup>10</sup> E. W. JOHNS, *Biochem. J.* **104**, 78 (1967).

<sup>11</sup> D. B. ROODYN, *Biochem. Soc. Symp.* **23**, 20 (1963).

<sup>12</sup> M. L. BIRNSTIEL, M. I. CHIPCHASE and W. G. FLAMM, *Biochim. biophys. Acta* **87**, 111 (1964).

<sup>13</sup> A. C. JENNINGS and R. K. MORTON, *Aust. J. biol. Sci.* **16**, 318 (1963).

<sup>14</sup> J. H. DUFFUS, *Biochim. biophys. Acta* **195**, 230 (1969).

<sup>15</sup> I. H. GOLDBERG, *Biochim. biophys. Acta* **51**, 201 (1961).

<sup>16</sup> A. TRAUB, E. KAUFMANN and Y. GINSBERG-TIETZ, *Expl Cell Res.* **34**, 371 (1964).

<sup>17</sup> J. H. RHO and M. I. CHIPCHASE, *J. Cell Biol.* **14**, 183 (1962).

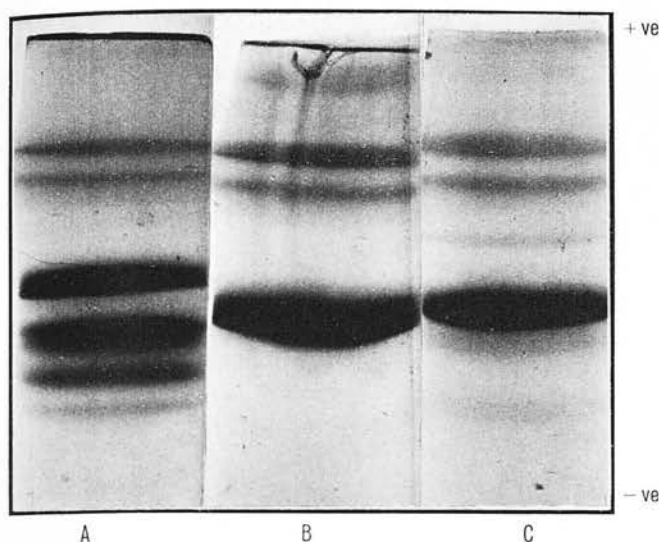


Fig. 2. Acrylamide gel electropherogram of A) calf thymus histones, B) nuclear histone, C) intact cell histones.

of greater and 1 of less mobility than the nuclear component.

The chemistry and biology of pea histones has been reviewed by SMITH *et al.*<sup>18</sup>. It was considered that while there may be no histone fraction specific to any pea tissue, reproducible quantitative differences may be observed in the histone fractions from different tissues<sup>19</sup>.

From the present work one must conclude that there appears to be only one histone present in endosperm nuclei at this stage in their development. This histone corresponds in mobility to histone II extracted from purified pea chromatin as described by SMITH *et al.*<sup>18</sup>.

The additional 3 components in the extract of whole cells may be either basic proteins, possibly ribosomal or, since the preparation almost certainly contains non-endosperm tissue, histones derived from embryo or seed coat nuclei. The mobility pattern of the whole cell histones is certainly similar to that associated with histones from pea tissue chromatin.

It may well be that the pattern for pea tissue chromatin is also a composite one derived from nuclei at different developmental stages. In the present case, however, the histone fraction was prepared from a fairly homogenous preparation of nuclei at the same developmental stage. Since endosperm nuclei disappear and presumably disintegrate after cell wall formation and amyloplast accumulation, the presence of a single histone may be related to their metabolic decline<sup>20, 21</sup>.

**Résumé.** Les noyaux d'endosperme ont été isolés de grains très jeunes d'orge. Le DNA, RNA et la protéine offraient entre eux les rapports 7:16:77. Les enzymes RNA polymérase (Nucléoside triphosphate: RNA nucléotidyle transférase, E.C. 2.7.7.6) et NAD pyrophosphorylase (ATP: NMN adényl transférase, E.C. 2.7.7.1) ont été observés dans les noyaux isolés. Un seul histone peut être trouvé dans la préparation des noyaux.

C. M. DUFFUS

Table II. Metabolic activity of isolated nuclei

Enzyme	Activity
RNA polymerase	nmoles of ( <sup>14</sup> C) ATP incorporated into RNA/mg protein/40 min
Normal system	2.7
0.05 ml ribonuclease (1 mg/ml) for 5 min after incubation	2.0
Nuclei + 0.05 ml deoxyribonuclease (1 mg/ml) for 20 min before incubation	0.5
NAD pyrophosphorylase	nmoles NAD/mg protein/30 min
	0.16

Each figure is the mean of 2 experiments.

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23 November 1971.

<sup>18</sup> E. L. SMITH, R. J. DELANGE and J. BONNER, *Physiol. Rev.* 50, 159 (1970).

<sup>19</sup> D. M. FAMBROUGH, F. FUJIMURA and J. BONNER, *Biochemistry* 7, 575 (1968).

<sup>20</sup> I thank Mrs. BOBBIE ROSIE for expert technical assistance.

<sup>21</sup> I thank the Agricultural Research Council for supporting this work and the Royal Society of London for a research microscope. Plant material was kindly supplied by the Scottish Society for Research in Plant Breeding. I am grateful to the Department of General Microbiology for the use of a Leitz photomicroscope.

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## Starch Hydrolysing Enzymes in the Developing Barley Grain

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**Summary.** The enzymes  $\alpha$ -amylase ( $\alpha$ -1, 4-glucan 4-glucanohydrolase, 3.2.1.1),  $\beta$ -amylase ( $\alpha$ -1, 4-glucan maltohydrolase, 3.2.1.2) and phosphorylase ( $\alpha$ -1, 4-glucan: orthophosphate glucosyltransferase, 2.4.1.1) were assayed in whole grains of barley throughout the maturation period.  $\alpha$ -amylase and phosphorylase had peaks of activity between 25 and 30 days after anthesis. On the other hand the activity of  $\beta$ -amylase in both the available and latent forms reached a maximum value at 35 days after anthesis which did not decrease thereafter.  $\beta$ -amylase activity was also assayed throughout development in the endosperm, aleurone, testa pericarp and embryo. Latent  $\beta$ -amylase reached a constant maximum value in endosperm at 35 days but available  $\beta$ -amylase reached a peak of activity at 25 days and then declined to zero at 45 days. Only latent  $\beta$ -amylase was associated with the aleurone layer and activity rose to a maximum value at 35 days. The testa pericarp had mainly latent  $\beta$ -amylase whose activity fell from an early maximum at 21 days to zero at 35 days. No hydrolytic activity was associated with the embryo. The phosphorylase activity was low and mainly associated with the endosperm fraction.

### Introduction

The presence of  $\alpha$ -amylase has been demonstrated in the developing barley grain (Duffus, 1969a; LaBerge *et al.*, 1971) and has been localised mainly in the endosperm and aleurone (Duffus, 1969a). While the presence of  $\beta$ -amylase in intact barley kernels has also been verified (LaBerge *et al.*, 1971) over the developmental period, little is known of the relative activities of enzymes of starch hydrolysis and of their location within the different tissues of the developing grain. Bilderback (1971) investigated the general amylolytic activity of the aleurone layer and starch endosperm of developing barley grain but the pattern of activity of the individual enzymes throughout maturation was not described.

The present paper describes the pattern of activity of several hydrolytic enzymes capable of using starch as substrate in intact grain throughout the maturation period. The relative activities throughout



maturation of the latent and available  $\beta$ -amylases are also described for the individual tissues of the grain.

### Materials and Methods

The two row barley, *Hordeum distichum* (L.) Lam. var. Maris Baldric was used. The conditions of growth and the method used to determine the date of anthesis were as described by Merritt and Walker (1969). The grain was used either fresh from the growing ear or after storage for periods of up to three months at  $-18^{\circ}\text{C}$ .

Whole grain was dehusked by hand before use leaving the testa pericarp as the outside layer and with the embryo still attached. The aleurone fraction was prepared by removing the testa pericarp and embryo from intact grains and separating the aleurone layer from the endosperm by gentle grinding in a glass homogeniser in the appropriate buffer. The resulting suspension was filtered through a double layer of muslin and the aleurone which was left behind washed several times. The endosperm fraction was that remaining after removal of testa pericarp, aleurone and embryo.

Enzymes were extracted by homogenising the various tissue fractions in the appropriate buffer. The supernatants obtained after centrifugation for 10 minutes in an MSE bench centrifuge at speed 10 were used as the source of enzyme. The concentration of tissue used varied from 40 grains or parts thereof in 2.0 ml of buffer at 2 days after anthesis to 10 grains in 4.0 ml at 35 days. Older material in general required a period of at least one hour following homogenisation for complete extraction of the enzyme.

$\alpha$ -amylase was assayed by the method of Briggs (1967). The tissue was extracted with 0.2% calcium acetate (pH 6.0) for one hour at room temperature, heated to  $70^{\circ}\text{C}$  for 20 minutes to inactivate  $\beta$ -amylase and cooled to  $0^{\circ}\text{C}$ . The clear supernatant after centrifugation was assayed for enzyme activity by the rate of decrease in starch-iodine colour at 608 m $\mu$ . The results were expressed in arbitrary units per grain as described by Duffus (1969b).

$\beta$ -amylase was assayed at pH 3.6 in the presence of ethylene diamine tetra acetic acid (EDTA). Under these conditions  $\alpha$ -amylase activity is inhibited (Blom *et al.*, 1936; Oikawa, 1959). Grains were homogenised in 0.1 M acetate buffer pH 3.6 and containing  $10^{-4}$  M EDTA. The suspension was left for one hour at  $0^{\circ}\text{C}$ , centrifuged and the clear supernatant assayed for  $\beta$ -amylase using starch as substrate as described above for  $\alpha$ -amylase. The measured enzyme activity was termed available  $\beta$ -amylase. This enzyme was also assayed by measuring the rate of release of maltose from starch at  $30^{\circ}\text{C}$  as described by Bernfeld (1951). For measurement of total  $\beta$ -amylase activity the acetate buffer contained 1% papain which can convert the enzyme from an inactive or latent state to the active or available form (Ford and Guthrie, 1908). The value for latent  $\beta$ -amylase activity was then obtained by subtracting that in the absence of papain from the total value which included both latent and available  $\beta$ -amylase.

An estimate of total available hydrolytic activity was obtained by homogenising grains in 0.2% calcium acetate pH 6.0, leaving for one hour at room temperature, centrifuging and assaying the clear supernatant for rate of disappearance of starch. The results were expressed as arbitrary units per grain as described above.



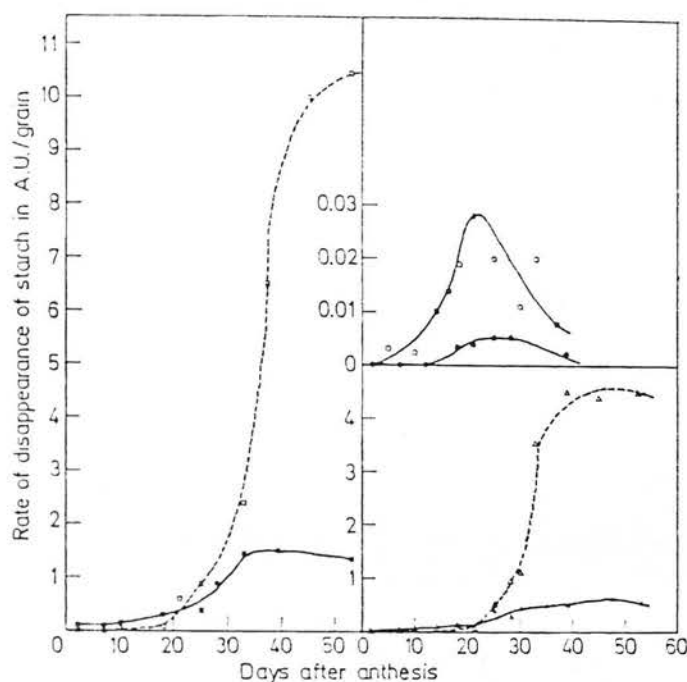


Fig. 1. Relative activities of starch hydrolysing enzymes in intact barley grains over the maturation period.  $\square$  Total latent hydrolytic activity;  $\blacksquare$  total available hydrolytic activity;  $\bullet$  phosphorylase;  $\circ$   $\alpha$ -amylase;  $\Delta$  latent  $\beta$ -amylase activity;  $\blacktriangle$  available  $\alpha$ -amylase activity

An estimate of total hydrolytic activity was obtained by including 1% papain in the calcium acetate solution.

Phosphorylase was assayed by the method of Cori *et al.* (1938), except that starch and not glycogen was used as substrate and results were expressed in arbitrary units as the rate of phosphate dependent starch disappearance.

### Results

Fig. 1 shows the variation in activity of the hydrolytic enzymes phosphorylase,  $\alpha$ -amylase, latent and available  $\beta$ -amylase and total latent and available hydrolytic activity with age of the whole grain. Activities were expressed as the rate of disappearance of starch in arbitrary units/grain. Both phosphorylase and  $\alpha$ -amylase reach a peak of activity between 20 to 30 days after anthesis.  $\alpha$ -amylase activity could be detected in the youngest grains but phosphorylase was only detectable by this method from 12 days after anthesis. Experiments using gel electro-

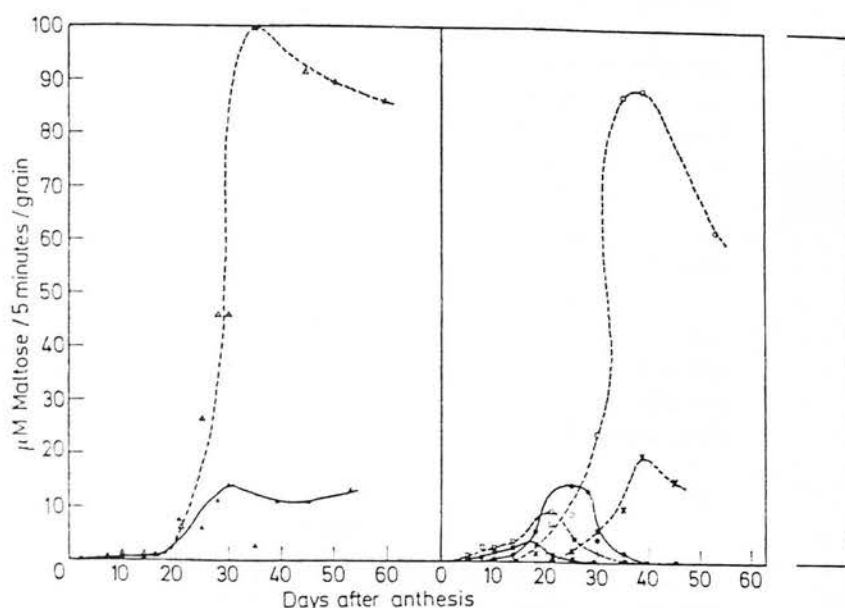


Fig. 2. Variation in available and latent  $\beta$ -amylase activity in different grain tissues throughout maturation. ● available enzyme in endosperm; ○ latent enzyme in endosperm; ■ available enzyme in testa pericarp; □ latent enzyme of testa pericarp; × latent enzyme in aleurone; ▲ available enzyme in intact grain; △ latent enzyme in intact grain

phoresis indicate that phosphorylase is present from 7 days after anthesis (Baxter, 1972). In comparison the latent and available  $\beta$ -amylases and total and available hydrolytic activity reached a steady maximum value at 30–35 days after anthesis. Initially available activity was slightly greater than latent enzyme activity. After 21 days latent activity became greater than available activity and increased dramatically while available activity remained at a comparatively low level.

While activities of the various enzymes are not strictly comparable as the conditions of assay varied, it is probably true to say that the  $\alpha$ -amylase and phosphorylase activities are small in comparison with the other two.

The pattern of activity of  $\beta$ -amylase activity in the different grain tissues throughout maturation is an interesting one and shows a succession of maxima (Fig. 2). Since the activity in this case was measured by maltose production from starch, results for intact grain were included for purposes of comparison. Activity first appeared in the testa pericarp. Available activity reached an early maximum value at 14–18 days;

latent activity which was ultimately greater than available activity reached a maximum value at 21 days. The next maximum value was available endosperm activity at 37 days and latent aleurone activity at about the same time. The activity in each tissue decreased after the maximum value had been reached. Both latent and available activity of testa pericarp and available activity of endosperm fell to zero around 35–40 days. In endosperm available  $\beta$ -amylase appeared first and was initially greater than that of the latent enzyme. In testa pericarp latent  $\beta$ -amylase activity was always greater than available enzyme activity; in aleurone no available activity could be detected at any time. No activity could be detected at any time in the embryo.

Both latent and available total hydrolytic activity and latent and available  $\beta$ -amylases were present in the soluble fraction remaining after centrifugation at  $40000 \times g$ . No activity was associated with the pellet which contained amyloplasts, mitochondria, cell walls and membranes.

### Discussion

Since we wanted to assess the relative importance of each enzyme in starch hydrolysis, methods were devised in which each was assayed by the rate of disappearance of starch. Thus instead of using the more specific method for  $\alpha$ -amylase with  $\beta$ -limit dextrin as substrate (Briggs, 1961), the enzyme was assayed under conditions in which other enzymes of starch hydrolysis were inactivated. Similarly  $\beta$ -amylase was assayed under conditions in which  $\alpha$ -amylase was inactive. The overall pattern of results obtained with this method is the same as that when the enzyme was assayed by the rate of appearance of maltose (Fig. 2). The results indicate that  $\beta$ -amylase is a very much more active enzyme than either  $\alpha$ -amylase or phosphorylase (Fig. 1). Furthermore, both sets of enzymes have quite different patterns of activity throughout maturation.  $\alpha$ -amylase and phosphorylase rise to a maximum value and thereafter decline. The  $\beta$ -amylases increase steadily throughout maturation reaching a steady maximum value. These results are consistent with the fact that  $\alpha$ -amylase (Varner, 1964) and therefore perhaps also phosphorylase is synthesised *de novo* in the germinating grain whereas  $\beta$ -amylase is released from a bound form (Weichherz and Asmus, 1931). LaBerge *et al.* (1971) found a similar pattern of activity throughout development for  $\alpha$ - and  $\beta$ -amylases.

In the intact grain (Fig. 1) and in endosperm (Fig. 2) available  $\beta$ -amylase activity is initially greater and appears earlier than latent  $\beta$ -amylase activity. It may therefore be that  $\beta$ -amylase is first synthesised in the

soluble form in endosperm, subsequently being converted to the latent form. In testa pericarp, however, latent  $\beta$ -amylase is greater than available  $\beta$ -amylase throughout development. In aleurone only the latent enzyme was detected. This appeared at about 20 days after anthesis. Thus the pattern of  $\beta$ -amylase synthesis in testa pericarp and aleurone is very different from that in endosperm. It may possibly involve the release of available enzyme from initially synthesised latent enzyme. Gibson and Paleg (1972) have reported that  $\alpha$ -amylase of wheat aleurone after treatment with gibberellic acid showed distinct structural latency, with mechanical or chemical treatments required to release the enzyme in an active form. The enzyme was localised within a membrane enclosed lysosome like organelle. It may be, therefore, that  $\beta$ -amylase of barley aleurone and testa pericarp has similar properties.

The existence of successive maxima in the tissues under investigation (Fig. 2) underlines their differences, and suggests that each one has a separate system controlling synthesis or release of the enzyme  $\beta$ -amylase and that they are activated in the order testa pericarp, endosperm and aleurone. It must be noted however, that since the results are expressed per grain, the levels of activity may reflect the amount of tissue present. For example at 5–10 days the greatest fraction by weight is testa pericarp, but at 30 days the greatest fraction is endosperm. Thus there may not be much difference in enzyme activity per cell. Preparations of green testa pericarp show some photosynthetic activity as measured by light dependent oxygen evolution. It may be that  $\beta$ -amylase is involved in the breakdown and transportation of carbohydrate from the chloroplasts for resynthesis of starch in the amyloplasts of the endosperm. The testa pericarp becomes yellow and dries out at about 28–30 days after anthesis and this process is accompanied at least in the isolated tissue by a decrease in  $\beta$ -amylase activity. In endosperm the rapid increase in available  $\beta$ -amylase activity is associated with a period of rapid starch deposition in amyloplasts (Baxter and Duffus, 1971). Perhaps the  $\beta$ -amylase in endosperm and aleurone generates polyglucan primers for the starch synthetase reaction. The order of appearance of successive maxima is similar to that of senescence in the respective tissues. Thus the relative activities of the  $\beta$ -amylases may reflect the age and degree of senescence of the given tissue.

The function of  $\alpha$ -amylase (if any) whose distribution among the different tissues has been previously described (Duffus, 1969a) may be similar to that postulated for  $\beta$ -amylase. Results with intact grain indicated that its synthesis may be controlled by gibberellic acid. Bilderback (1971) has reported otherwise, but since total 'amylase'

activity and not  $\alpha$ -amylase—which is a small fraction of the total activity—was assayed by him, this observation is of doubtful significance. Furthermore this author claimed to be able to measure 'amylase' activity in isolated 5 day old aleurone layers. Since this is around the period of free nuclear division (Thompson and Johnston, 1945) a complete aleurone layer may not then have been formed. It is doubtful also that significant results could be obtained from as few as 5 aleurones/ml since at this stage the grains may be as little as 2–3 mm in length. Preparations, therefore, may have been contaminated with green testa pericarp.

Results in terms of days of anthesis may not of course be strictly comparable. The maturation period in Scotland is twice that in North America. Under greenhouse conditions, however, the difference is much less — 40–50 in North America and 55–65 in Scotland.

The probable functions and properties of barley endosperm phosphorylase are currently under investigation in this laboratory.

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## ENZYMES OF CARBOHYDRATE METABOLISM IN DEVELOPING *HORDEUM DISTICHUM* GRAIN

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**Key Word Index**—*Hordeum distichum*; Gramineae; barley; endosperm; amyloplast; carbohydrate metabolism; GIP synthesis.

**Abstract**—Variations in activity of several enzymes associated with carbohydrate metabolism were recorded during the development of barley endosperm. The enzymes investigated were: sucrose-UDP (ADP) glucosyl transferase; invertase; UDPG (ADPG) pyrophosphorylase; hexokinase; glucose-6-phosphate ketoisomerase; phosphoglucomutase, and nucleosidediphosphokinase.

### INTRODUCTION

INITIATION of short-chain glucan primers may be carried out by phosphorylase (E.C. 2.4.1.1), with glucose-1-phosphate (GIP) serving as the glucosyl donor.<sup>1,2</sup> The nucleotide sugars, UDPG (UDP-glucose) and ADPG (ADP-glucose) which, with these primers, are the substrates for starch synthesis<sup>3,4</sup> may be synthesized from sucrose by sucrose-UDP (ADP) glucosyl transferase (E.C. 2.4.1.13),<sup>5</sup> or from GIP by ADPG (UDPG) pyrophosphorylase (E.C. 2.7.7.9).<sup>6</sup> GIP, itself a product of sucrose metabolism, is thus a key intermediate in the conversion of sucrose to starch, and variations in its concentration may provide a regulatory mechanism in starch biosynthesis. Enzyme systems concerned in the biosynthesis and metabolism of GIP and the nucleotide sugars were therefore investigated in barley endosperm, in order to determine changes in activity during maturation.

### RESULTS

#### *Major Biochemical Constituents*

The relative contributions of starch, reducing sugars, and protein to the dry weight of the grain, and of water to the fresh weight, are shown in Fig. 1. The proportion of reducing sugars fell as starch accumulated in the endosperm. Protein levels increased during the first 20 days after anthesis, and then remained fairly constant during maturation. Water content was high in the young grain, reaching a maximum around 14 days after anthesis, then falling steadily as the grain ripened. The levels of biochemical constituents described here were approximately the same as those in the grain used for enzyme assays (see below).

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<sup>5</sup> DELMAR, D. P. and ALBERSHEIM, P. (1970) *Plant Physiol.* **45**, 788.

<sup>6</sup> TURNER, J. F., TURNER, D. H. and LEE, J. B. (1957) *Australian J. Biol. Sci.* **10**, 407.



*Sucrose-UDP (ADP) Glucosyltransferase*

Activity with UDP, which was considerably greater than with ADP in soluble endosperm extracts, could be detected at 5–7 days, and increased rapidly until 25 days (Fig. 2). Activity with ADP was not apparent until 8 days, and rose gradually to a maximum around 34 days. No activity with either UDP or ADP was detected in amyloplast fractions.

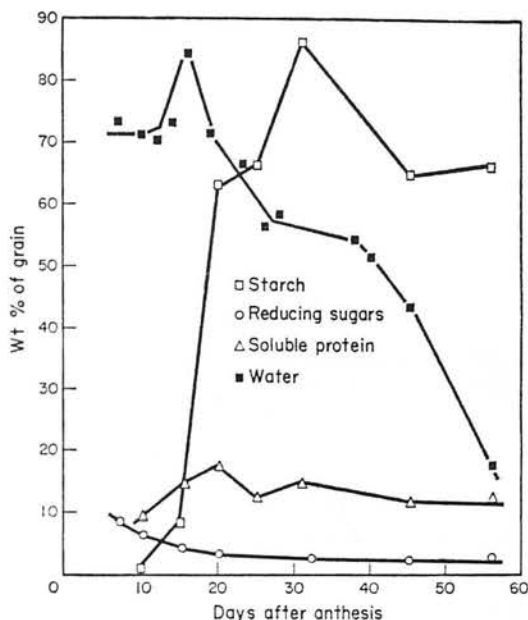


FIG. 1. STARCH, SOLUBLE REDUCING SUGARS AND TOTAL SOLUBLE PROTEIN AS A % OF DRY WT, AND WATER AS A % OF FR. WT OF WHOLE ENDOSPERM DURING MATURATION.

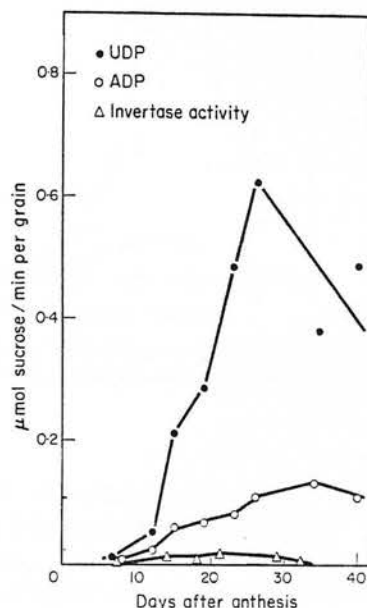


FIG. 2. SUCROSE—UDP (ADP) GLUCOSYL TRANSFERASE ACTIVITY WITH UDP AND ADP, AND INVERTASE ACTIVITY IN SOLUBLE ENDOSPERM EXTRACTS OF DEVELOPING ENDOSPERM.

*Invertase (E.C. 3.2.1.26)*

This was measured both at pH 4.8 and 7.2, and activity was low in each case. The variation in activity at pH 7.2 throughout maturation in the soluble fraction of endosperm is shown in Fig. 2. Activity was detected around 10 days after anthesis and remained fairly constant during maturation, declining after 30–34 days. By 21 days after anthesis a significant proportion of the total grain activity was located in the soluble fraction of the embryo.

*UDPG (ADPG) Pyrophosphorylase*

The UDPG enzyme could be detected in the soluble endosperm fraction by 7 days after anthesis. Activity increased rapidly at first, levelling off around 16 days, then rising again to a peak at 24 days (Fig. 3). The ADPG enzyme appeared somewhat later—around 12 days, and a maximum activity, slightly lower than that of the UDPG enzyme, was reached by 21 days.

*Inorganic Pyrophosphatase (E.C. 3.6.1.1)*

Figure 4 shows that activity in the early stages of maturation closely resembled the pattern of ADPG pyrophosphorylase. Maximum activity, however, was attained around the same

period as UDPG pyrophosphorylase. Activity was chiefly located in the soluble fraction, but some could be detected in the amyloplasts from 12 days after anthesis.

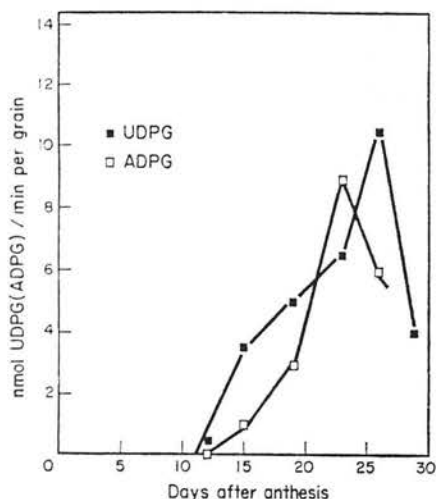


FIG. 3. UDPG AND ADPG PYROPHOSPHORYLASE ACTIVITY IN SOLUBLE ENDOSPERM EXTRACTS.

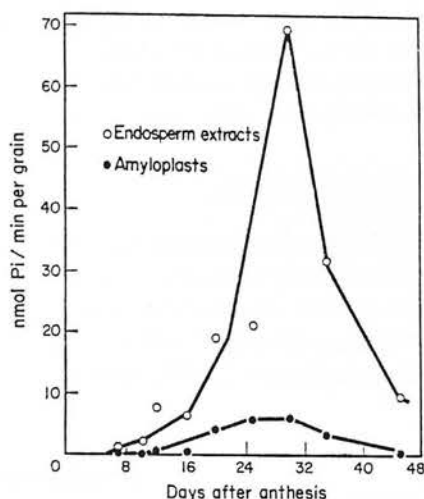


FIG. 4. INORGANIC PYROPHOSPHATASE ACTIVITY IN SOLUBLE ENDOSPERM EXTRACTS AND AMYLOPLASTS.

#### Hexokinase (E.C. 2.7.1.1)

This enzyme was present in both soluble and amyloplast fractions from 7 days after anthesis and increased steadily throughout the initial stages of maturation, levelling off around 21 days. Similar results were obtained with glucose and fructose as substrates. The results expressed in Fig. 5 were obtained using glucose. Amyloplast activity was approximately half that of the soluble fraction.

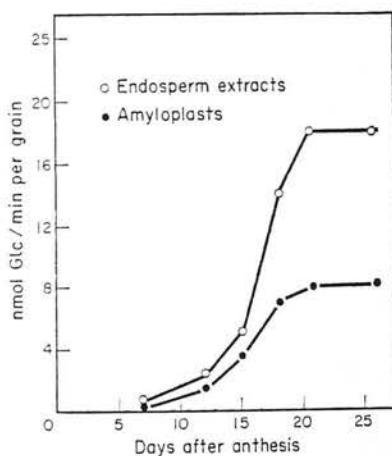


FIG. 5. HEXOKINASE ACTIVITY IN SOLUBLE ENDOSPERM EXTRACTS AND AMYLOPLASTS.

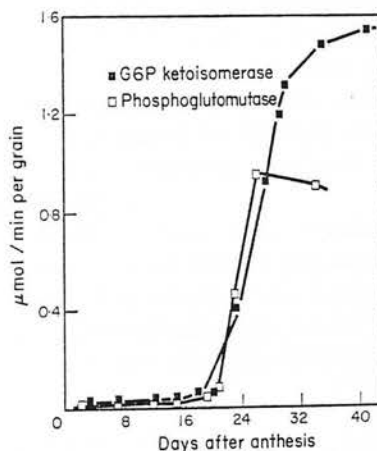


FIG. 6. G6P KETOISOMERASE ACTIVITY IN  $\mu\text{mol F6P/min/10 grains}$  AND PHOSPHOGLUCOMUTASE IN  $\mu\text{mol P}_i\text{/min/10 grains}$  (VALUES  $\times 2$ ) IN SOLUBLE ENDOSPERM EXTRACTS.

*Phosphoglucomutase* (E.C. 2.7.5.1) and *Glucose-6-phosphate ketoisomerase* (E.C.5.3.1.9)

Both enzymes were present in the soluble endosperm extract very early in development, and exhibited similar patterns of activity (Fig. 6). There was no appreciable change until 18 days, when activity increased sharply, between 20 and 22 days. Glucose-6-phosphate (G6P) ketoisomerase activity began to level off around 25 days, when phosphoglucomutase was already declining.

*Nucleoside diphosphokinase* (E.C. 2.7.4.6)

Sucrose-UDP glucosyl transferase and ADPG pyrophosphorylase exhibit their greatest increase in activity around 15–20 days after anthesis. Nucleoside diphosphokinase was therefore assayed in soluble endosperm extracts of this age to determine whether interconversion of the nucleotides formed in these reactions was possible. The presence of the enzyme was confirmed, and it was found to be more active with UTP (7.5 nmol/min/grain) than with GTP (4.5 nmol/min/grain).

#### DISCUSSION

Patterns of changes in reducing sugars closely resembled those recorded in wheat<sup>7</sup> and peas,<sup>8</sup> and are consistent with a mechanism by which starch is synthesised from a precursor pool of soluble reducing sugars. That is, the pool is initially depleted during the onset of rapid starch synthesis but is subsequently maintained at a constant (but lower) level, presumably by the continuing supply of sucrose to the grain.<sup>7</sup> The increase in protein levels which immediately precedes the period of rapid starch accumulation may reflect synthesis of enzyme protein, since an increase in the activity of most enzymes investigated was observed during this stage of development. However, maintenance of protein concentrations during later maturation may be attributed to the accumulation of protein bodies in the starchy endosperm.<sup>9</sup>

The initial stages of starch synthesis, namely the formation of GIP and nucleotide sugars from translocated sucrose, appear to be localized in the soluble fraction of the endosperm, rather than in the amyloplasts. The relatively high activity of sucrose-UDP glucosyl transferase in young endosperm suggests that most of the sucrose entering the endosperm is converted to nucleotide sugars, predominantly UDPG. This contrasts with the situation in maize, where sucrose-UDP glucosyl transferase cannot be detected until 12 days after anthesis.<sup>10</sup> In this case sucrose is thought to be metabolized to glucose and fructose via invertase, at least in the early stages of development.<sup>11</sup>

Fructose is also a product of the sucrose-UDP glucosyl transferase reaction, and may be converted to GIP via hexokinase, G6P ketoisomerase and phosphoglucomutase activity,<sup>12,13</sup> since these enzymes are all present in barley endosperm soon after anthesis. In the young endosperm GIP synthesized in this way may be immediately polymerized by phosphorylase activity, forming short-chain  $\alpha$ -1,4-glucans which could serve as primers for transglucosylase activity.<sup>1,2,14</sup>

<sup>7</sup> JENNINGS, A. C. and MORTON, R. K. (1963) *Australian J. Biol. Sci.* **16**, 318.

<sup>8</sup> TURNER, J. F. (1969) *Australian J. Biol. Sci.* **22**, 1145.

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<sup>10</sup> TSAI, C. Y., SALAMINI, F. and NELSON, O. E. (1970) *Plant Physiol.* **46**, 299.

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<sup>12</sup> DE FEKETE, M. A. R. and CARDINI, C. E. (1964) *Arch. Biochem. Biophys.* **104**, 173.

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<sup>14</sup> BIRD, I. F. (1969) Ph.D. Thesis, University of London.

The diagram illustrates the sucrose cycle, a metabolic pathway for the conversion of sucrose to starch and back. The cycle is numbered 1 through 8:

- 1:** Sucrose is converted to UDPG.
- 2:** UDPG is converted to UTP.
- 3:** UDPG is converted to Starch.
- 4:** UTP is converted to ADP.
- 5:** ADP is converted to GDP.
- 6:** ADPG is converted to ATP.
- 7:** ATP is converted to ADP.
- 8:** ADPG is converted to Starch.

The cycle is coupled with the hydrolysis of GTP to GDP, which provides energy for the conversion of UDPG to UTP (step 2) and ADPG to ATP (step 6). The diagram also shows the conversion of ADP to ATP (step 7) and the conversion of ATP to ADP (step 4).

1. Sucrose-UDP glucosyl transferase
2. UDPG pyrophosphorylase
3. UDPG-starch synthetase
4. Nucleoside diphosphokinase
5. Starch phosphorylase
6. ADPG pyrophosphorylase
7. Inorganic pyrophosphatase
8. ADPG-starch synthetase

## EXPERIMENTAL

*Preparation of soluble endosperm extracts and amyloplast fractions.* The husk and testa-pericarp were removed by hand and the remaining endosperm (including the aleurone layer) together with the embryo constituted the 'whole grain' subsequently referred to. This must be distinguished from the 'whole grain' of several other workers (for example, Jennings and Morton<sup>7</sup>) which includes the testa-pericarp. To prepare soluble endosperm extracts, the 'whole grain' was suspended in the appropriate buffer at 4° and homogenized by hand in an all-glass homogenizer. The homogenate was filtered through muslin to remove cell debris and centrifuged at 4° for 10 min at 10 000 *g*. The supernatant solution formed the soluble endosperm extract. The pelleted material was washed once with buffer, re-centrifuged and re-suspended in buffer. This fraction was composed mainly of amyloplasts and is referred to as the amyloplast fraction. It was routinely examined by light microscopy and the age of the grain correlated with amyloplast diameter. The number of grains used for extraction was varied from 60 (at 2-3 days) to 10 (18 days onwards) per 2 ml buffer. The buffer required in the subsequent assay system was used as the extraction medium in each case.

<sup>16</sup> MERRITT, N. R. and WALKER, J. T. (1969) *J. Inst. Brewing* **75**, 156.

*Chemical analyses.* Reducing sugars,<sup>17,18</sup> carbohydrate,<sup>19</sup> and soluble protein<sup>20</sup> were measured by standard methods.

*Enzyme assays.* Sucrose-UDP glucosyl transferase<sup>21</sup> and invertase,<sup>10</sup> were assayed by increase in reducing sugars. ADPG (UDPG) pyrophosphorylase,<sup>8</sup> hexokinase,<sup>10</sup> and nucleoside diphosphokinase<sup>22</sup> were estimated spectrophotometrically by coupling a product of the reaction to the G6P dehydrogenase reaction.<sup>8</sup> Inorganic pyrophosphatase,<sup>23</sup> phosphoglucomutase<sup>10</sup> and G6P ketoisomerase,<sup>10</sup> were estimated by standard colorimetric methods.

*Acknowledgements*—The authors wish to thank the Scottish Plant Breeding Station, Pentlandsfield, East Lothian, for supplying the plant material. The microscope, a Vickers M15C, was purchased with a Royal Society grant. The work was supported by the Agricultural Research Council.

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<sup>18</sup> NELSON, N. (1944) *J. Biol. Chem.* **153**, 375.

<sup>19</sup> MORRIS, D. L. (1948) *Science* **107**, 254.

<sup>20</sup> LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265.

<sup>21</sup> MILNER, Y. and AVIGAD, G. (1964) *Israel J. Chem.* **2**, 316.

<sup>22</sup> DICKINSON, D. B. and DAVIES, M. D. (1971) *Plant Cell Physiol.* **12**, 157.

<sup>23</sup> HEPPEL, L. A. (1955) in *Methods in Enzymology* (COLOWICK, S. P. and KAPLAN, N. O., eds.), Vol. II, p. 570, Academic Press, New York.

## Short Communication

# Starch Synthetase: Comparison of UDPG and ADPG as Glucosyl Donors in Immature Barley Endosperm

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**Summary.** The activity of starch synthetase in developing barley endosperm was measured in amyloplasts and in the soluble endosperm fraction by incorporation of radioactively labelled glucose into starch. Both uridine diphosphate glucose (UDPG) and adenosine diphosphate glucose (ADPG) were used as glucosyl donors. Enzyme activity was initially located in the soluble fraction, but increasing activity could be detected in the amyloplast fraction during endosperm maturation.

Starch synthetase which catalyses the transfer of glucose from UDPG and ADPG to a glucan primer exists either as the free enzyme or bound to starch granules (de Fekete *et al.*, 1960). For higher plants, starch synthesis is generally faster with ADPG than with UDPG (Recondo and Leloir, 1961). It has been observed, however, that different physical forms of the enzyme (*i.e.* whether bound or free) frequently exhibit different specificities towards their glucosyl donors (Tanaka and Akazawa, 1968).

The work described in this paper compares the abilities of bound and free forms of starch synthetase from developing barley endosperm to utilise both UDPG and ADPG as glucosyl donors.

The two row barley *Hordeum distichum* (L) Lam. c. v. Maris Baldric was used throughout. Conditions of growth, methods used to determine the age after anthesis, and the extraction of amyloplast and endosperm fractions were as described previously (Baxter and Duffus, 1971, 1973). Starch synthetase was assayed by the incorporation of [<sup>14</sup>C]glucose into starch from uridine-5'-diphosphate (glucose-C<sup>14</sup>-(U)) (ADPG\*) by the method of Leloir *et al.* (1961). The UDPG\* (specific activity 233 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, and the ADPG\* (specific activity 75 mCi/mmol) was a gift from Dr. I. F. Bird (Rothamsted Experimental Station, Harpenden, Herts, U.K.).

Reaction mixtures contained 0.025  $\mu$ Ci UDPG\* (0.0225  $\mu$ Ci ADPG\*), together with 0.3  $\mu$ moles unlabelled UDPG (ADPG), 0.1 ml of the appropriate endosperm

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Table 1. UDPG-starch synthetase in soluble and amyloplast fractions of developing endosperm

Age in days after anthesis	Activity (cpm/g) <sup>a</sup>	
	Soluble fraction	Amyloplast fraction
7	17	8
18	91	41
22	217	592

<sup>a</sup> Results are averages of 3 determinations, corrected for background radiation and for non-enzymic controls.

fraction in 0.05 M glycine buffer, pH 8.3, and 0.05 ml of a 2.5% solution of soluble starch. The total reaction volume was 0.5 ml. Samples were incubated for 4 h at 37°C, then solid starch added as a carrier and the mixture centrifuged for 10 min at 13000  $xg$  to collect insoluble material. This was thoroughly washed to remove unreacted substrates, and the residual radioactivity determined using a Beckman liquid scintillation system. Samples were corrected for background radiation and for controls incubated without endosperm extract.

Table 1 illustrates the distribution of starch synthetase in amyloplast and soluble endosperm fractions, using UDPG\* as a glucosyl donor. At 7 days after anthesis, enzyme activity was low, and mainly located in the soluble fraction. A 5-fold increase in activity had occurred by 18 days after anthesis, although distribution between the fractions remained unchanged. By 22 days however, a rapid increase in activity had occurred and whereas, that in the soluble fraction had doubled, amyloplast-bound activity had increased by a factor of 10.

The comparative abilities of UDPG and ADPG to act as glucosyl donors for soluble and amyloplast fractions of 20-day endosperm are shown in Table 2. The results indicate that soluble starch synthetase was ten times more active with UDPG\* than with ADPG\*, while the reverse was true for amyloplast bound enzyme.

These results agree with previous work (Baxter and Duffus, 1971) which indicated that the bulk of the starch in barley endosperm was synthesised by amyloplast-bound starch synthetase using ADPG as a glucosyl donor. In young endosperm, however, this enzyme appeared to be more active with UDPG. The present study shows that in young endosperm most of the starch synthetase activity is in solution and UDPG linked. It seems likely that much of the bound enzyme prepared from young (4-10 day) endosperm represents free enzyme attached to the outer membranes of the amyloplasts, which contain little starch at this stage. As more starch is synthesised the starch-enzyme complex



Table 2. UDPG and ADPG as glucosyl donors for starch synthetase activity in 20 day endosperm extracts

Glucosyl donor	Activity (cpm/g) <sup>a</sup>	
	Soluble fraction	Amyloplast fraction
UDPG*	299	109
ADPG*	27	1273

<sup>a</sup> Results are averages of at least four determinations. Corrections as for Table 1.

will accumulate. The physical nature of bound starch synthetase may alter during endosperm development as the starch content of the amyloplasts increases. Thus it may be that the free enzyme utilises mainly UDPG, becoming more ADPG specific as it is increasingly complexed with starch. The free enzyme itself however, remains UDPG linked. Frydman and Cardini (1967) also suggested that bound and free starch synthetases were modifications of the same enzyme, by formation of an insoluble starch enzyme complex. They examined bound starch synthetases from several plant sources and concluded that, whereas reserve-starch accumulating tissues could utilise both glucosyl donors only ADPG was active with leaf enzymes. Since the free enzyme from spinach leaves is also ADPG specific, while the bound spinach seed enzyme is active with both UDPG and ADPG (Tanaka and Akazawa, 1968) it would appear that substrate specificity of starch synthetase is also tissue related. It is probable that, whereas leaf synthetases are ADPG specific whether bound or free, seed synthetases can utilise both UDPG and ADPG in proportions which depend upon the extent to which the enzyme is complexed with starch.

Alternatively it is also possible that the bound and free starch synthetases are isoenzymes with different substrate requirements. Multiple forms of starch synthetase have been isolated from waxy and non waxy strains of rice (Tanaka and Akazawa, 1971) and maize (Ozbun *et al.*, 1971). However these were prepared from the free enzyme only and were specific for ADPG, although different primer requirements were observed. There is no evidence as yet for multiple forms of bound starch synthetase.

The persistence of UDPG linked mechanisms in developing seeds may be concerned with the synthesis of endosperm cell wall components. Pectin and cellulose are found in the husk and embryo but in the endosperm the cell walls are composed almost entirely of structural protein, pentosans and  $\beta$ -glucans. UDPG has been implicated in the synthesis

both of pentosans (via UDP-D-xylose and UDP-L-arabinose) and of  $\beta$ -(1 $\rightarrow$ 3) and mixed  $\beta$ -(1 $\rightarrow$ 3),  $\beta$ -(1 $\rightarrow$ 4) linked polymers (Nikaido and Hassid, 1971). The glucose donor for cellulose biosynthesis, on the other hand, appears to be guanosine diphosphate glucose (Elbein *et al.*, 1964).

Ordin and Hall (1968) observed that with *Avena* coleoptiles, the proportions of  $\beta$ -(1 $\rightarrow$ 3) and  $\beta$ -(1 $\rightarrow$ 4) links formed *in vitro* were related to the concentration of UDPG, with  $\beta$ -(1 $\rightarrow$ 3) linked polymers predominating at higher UDPG concentrations. Thus, competition for UDPG with the pathways of endosperm cell wall synthesis may be an important regulatory factor in the early stages of starch biosynthesis in immature barley endosperm.

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## Some Enzyme Activities Associated with the Chlorophyll Containing Layers of the Immature Barley Pericarp\*

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**Summary.** Some photosynthetic and biochemical properties of the chlorophyll containing layers of the pericarp of developing barley have been investigated. The tissue changes from pale green to bright green early in development, chlorophyll disappearing only at the later stages of maturity. It contains chloroplasts and probably amyloplasts and starch bearing chloroplasts. It is capable of high rates of light dependent oxygen evolution. It has been shown that the enzyme phosphoenol pyruvate carboxylase (EC 4.1.1.31) is present in the pericarp and is 100 times as active in carbon dioxide fixation as ribulose diphosphate carboxylase (EC 4.1.1.39). Other enzymes present in the pericarp are phosphoenol pyruvate synthetase, pyrophosphatase (EC 3.6.1.1), malate NAD and NADP dehydrogenases (EC 1.1.1.37), malic enzyme (EC 1.1.1.40), and fructose 1,6 diphosphatase (EC 3.1.3.11).

### Introduction

The pericarp is one of a number of morphologically distinct layers of cells surrounding the cereal grain endosperm and embryo. Part of it consists of chlorophyll containing parenchyma cells which are 1–2 cells thick over most of the grain and several layers thick in the region where the grain is grooved. This layer accounts for the bright green colour of the immature barley grain.

The structure of the pericarp and those layers associated with it such as the testa and aleurone has been well described (Bergal and Clemencet, 1962). On the other hand little is known of the biochemical function of the pericarp and whether or not it can contribute by means of photosynthesis to the accumulation of storage materials in the developing grain.

Archbold (1942) has shown that the dry matter entering the barley grain is derived from photosynthesis after ear emergence. The contri-

\* Abbreviations: RDP, Ribulose 1,5-diphosphate; PEP, phosphoenol pyruvate.

bution of photosynthesis by the ear to the grain may account for as much as 76%—in terms of mg carbon dioxide fixed (Frey-Wyssling and Buttrose, 1959). This will have been contributed by photosynthesis in the awns, glumes and possibly the pericarp. Buttrose and May (1959) have shown that intact ears, when supplied with  $^{14}\text{CO}_2$  or  $[^{14}\text{C}]$ sucrose early in maturity accumulate radioactivity in the pericarp. The results did not distinguish between carbon fixed in the other tissues of the ear and transported to the pericarp, and carbon fixed in the pericarp itself.

More recently it has been shown (Evans and Rawson, 1970) that in awned varieties of wheat, photosynthesis by the grain itself can account for as much as 34% of gross photosynthesis in the intact ear. In addition there is some evidence (Carr and Wardlaw, 1965) that excised wheat grains can assimilate externally applied  $^{14}\text{CO}_2$  in the light. It was thought likely that respired carbon dioxide rather than atmospheric carbon dioxide would be fixed by this route. The photosynthetic activity of the grain is presumably located in the chlorophyll containing layers of the pericarp.

The present work then describes some of the biochemical and photosynthetic properties of this layer in immature barley grain.

#### Materials and Methods

Two row barley plants [*Hordeum distichum* (L.) Lam. cv. Julia] were used. Conditions of growth and methods used to determine the date of anthesis were as described by Merritt and Walker (1969).

*Light Microscopy.* Intact pericarp tissue and tissue homogenates (q.v.) in 10% sucrose were examined under phase contrast or with direct light after staining with  $\text{I}_2/\text{KI}$  solution

*Tissue Preparation* Pericarps were isolated as follows. First, the glumes and outer transparent layer of pericarp were removed from the grain. The thin green layer was then removed, any adhering testa or aleurone scraped off, and the residual tissue dropped into a suitable volume of an appropriate buffer. This operation was carried out as quickly as possible since the tissue dehydrates quickly. Chloroplasts were isolated by homogenising at least 20 pericarps in 1–2 ml of the assay medium described by Walker *et al.* (1968). After filtering through muslin the homogenate was centrifuged at  $1000 \times g$  for 3 min. The pellet, which contained amyloplasts and mitochondria as well as chloroplasts, was used for the measurement of light dependent oxygen evolution (Hill reaction) at  $25^\circ\text{C}$  in a Rank oxygen electrode (Walker *et al.*, 1968) with dichlorophenol indophenol as electron acceptor (Trebst, 1972).

*Assays.* 10 pericarps in 1.0 ml of the appropriate buffer were homogenised in an all glass Potter type homogeniser and enzyme activity was assayed either directly in this solution or in the supernatant after centrifugation at  $10000 \times g$  or  $25000 \times g$  for 10 min. Chlorophyll from the whole homogenate was extracted into 80% acetone within 30 sec of homogenisation as some phenol oxidation (prevented to some extent by the addition to the grinding medium of 10 mM mercaptoethanol)

takes place. The method was adjusted to optimize the measurement of chlorophyll concentration to avoid artificially high estimates of enzyme activity, which is expressed per mg of chlorophyll. Chlorophyll was estimated by the method of Arnon (1949). RDP carboxylase was assayed in the tissue homogenate by measuring the RDP dependent incorporation of  $^{14}\text{CO}_2$  into acid stable material as described by Slack and Hatch (1967). To overcome the lag in reaction rate the reaction mixtures were pre-incubated at  $0^\circ\text{C}$  for 10 min without added substrate. The acidified reaction mixture was centrifuged and the supernatant (made up to 0.5 ml with distilled water) was counted for 10–20 min in 5.0 ml of dioxan based liquid scintillator (N.E. 220; Nuclear Enterprises, Edinburgh) in a Beckmann Liquid Scintillator System. Samples were automatically corrected for background radiation.

PEP carboxylase was determined in the tissue homogenate as described by Slack and Hatch (1967). The acidified reaction mixtures were counted as described above.

PEP synthetase was assayed in the  $10000\times g$  supernatant using the reaction conditions of Hatch and Slack (1968). The disappearance of pyruvate and the appearance of PEP were measured using pyruvate kinase and ADP as described by Cooper and Kornberg (1965). The complete stoichiometry of the reaction was not verified. Inorganic pyrophosphatase was assayed in the tissue homogenate by the method of Heppel (1955) but using 0.05 M Tris-maleate buffer pH 7.2. The phosphate released was measured by the method of Allen (1940). Malic enzyme was assayed in the  $25000\times g$  supernatant as described by Ochoa *et al.* (1948). Malate NAD and NADP dehydrogenases were assayed in the  $25000\times g$  supernatant as described by Mehler *et al.* (1948). Fructose 1,6 diphosphatase was assayed as described by Racker and Schroeder (1958) and the phosphate released measured as described above.

## Results

Preparations of intact tissues and tissue homogenates contained both amyloplasts and chloroplasts. Unlike those found in starchy endosperm the amyloplasts were of similar size and about  $4\text{ }\mu\text{m}$  in diameter. The amyloplasts are probably associated with the pericarp tissue itself. That they originate from contamination by another subepidermal layer cannot be completely ruled out. However, preliminary examination of the tissue by electron microscopy (J. M. Chappell, unpublished) indicates the presence within the cells of both chloroplasts and starch containing chloroplasts (Amyloplasts).

The variation in chlorophyll content of the pericarp with age in days after anthesis is shown in Fig. 1. Chlorophyll is present throughout the developmental period and increases rapidly over the period 14–21 days to reach a maximum value around 27 days. All subsequent measurements of activity, therefore, were made on pericarp tissue from grain aged between 14 and 27 days after anthesis.

The activities of the various enzyme systems expressed in  $\mu\text{moles/mg}$  of chlorophyll/min and in  $\mu\text{moles/10 pericarps/min}$  are shown in Table 1. Since activity generally varies with age after anthesis and since deter-

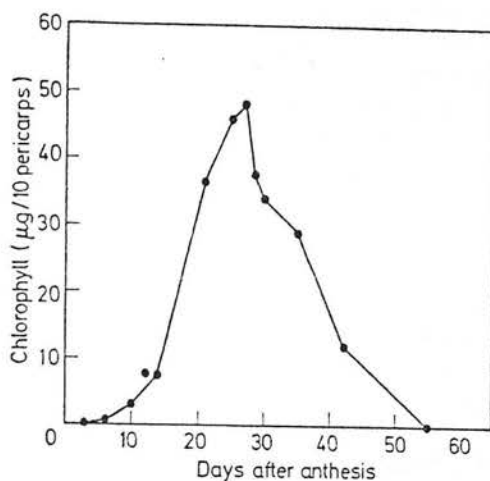


Fig. 1. Variation in chlorophyll content of the pericarp with age in days after anthesis

Table 1. Some enzyme activities associated with the chlorophyll containing layers of the immature barley pericarp

	$\mu\text{moles/mg of chlorophyll/min}$	$\mu\text{moles/10 pericarps/min}$
Light dependent oxygen evolution (Hill reaction)	0.1-1.9	0.006-0.034
RDP carboxylase	0.2-0.3	0.003-0.008
PEP carboxylase	3.5-32	0.13 -0.53
PEP synthetase	4-22	0.04 -0.76
Inorganic pyrophosphatase	1.1-5.4	0.05 -0.30
Malic NAD dehydrogenase	40-450	3.7 -7.5
Malic NADP dehydrogenase	0.2-3.3	0.018-0.17
Malic enzyme	0.4-2.5	0.014-0.018
Fructose, 1,6-diphosphatase	13-34	0.81-1.35

minations were made on samples of different ages after anthesis, a wide range of activity is shown. The figures reported therefore extend from the minimum activity (generally around 14 days after anthesis) to the maximum measureable activity (generally around 21-23 days after anthesis).

The rate of light dependent oxygen evolution varied throughout maturation, being generally higher in the later stages of development. The rates were low in comparison with those of the other enzymes.

The activities of both PEP and RDP carboxylases were linear over the periods (10 and 20 min respectively) of measurement. The results reported are derived from the slope of the graph of incorporation of substrate vs. time rather than from single measurements. The activity of RDP carboxylase was much less than that of PEP carboxylase. About 5–10% of total PEP carboxylase was associated with the (unwashed) chloroplast fraction.

An enzyme system catalysing the ATP dependent disappearance of pyruvate with the concomitant production of PEP was present in the pericarp. Since the complete stoichiometry of the reaction was not verified conclusions based on these results are tentative. Some activity was recorded in the absence of added ATP. Some 10% of the enzyme activity was associated with the (unwashed) chloroplast fraction. Both pyrophosphatase and fructose 1,6-diphosphatase were present in whole homogenates of pericarp. Again, some activity was associated with the chloroplast and amyloplast fraction.

Both NAD and NADP linked malate dehydrogenases were present in the pericarp, activity with NADP being much less at all ages of pericarp development than with NAD. Malic enzyme was present in measurable quantity throughout the age range investigated.

### Discussion

The developmental pattern of chlorophyll concentration per grain follows the general one recorded for overall changes throughout development (Baxter and Duffus, 1973). That pericarp chloroplasts are active in photosynthesis is shown by their ability to catalyse a Hill reaction. The rates were rather higher than those reported by Cockburn (1967) ( $0.1 \mu\text{M O}_2/\text{min}/\text{mg}/\text{chlorophyll}$ ) for a Hill reaction with spinach chloroplasts.

The results, expressed in  $\mu\text{moles}/\text{mg}$  of chlorophyll/min are intended only for comparison with those of other workers in the field. Since most of the enzymes described, with the exception of Hill activity and RDP carboxylase, do not have a definite association with the chloroplast this method of expressing enzyme activities is largely meaningless. In addition such figures do not reflect the absolute levels of enzyme activity since the chlorophyll content/pericarp varies with age after anthesis. Absolute levels of enzyme activity expressed in  $\mu\text{moles}/10$  pericarps/min are therefore included in the results.

The activity of RDP carboxylase was lower by a factor of 10 than activities recorded by other workers for cereal leaves (Slack and Hatch, 1967). Interpretation of this data is difficult since this enzyme is inhibited



by the oxidation products of phenolic compounds (Baldry *et al.*, 1970). Phenol oxidase enzymes are present in chloroplasts (Tolbert, 1973) and may be present in the pericarp of barley. The activity of PEP carboxylase was 50–100 fold greater than that recorded for wheat or barley leaves and was of the order described for plants such as sugar cane, maize and sorghum, all of which appear to use this enzyme in preference to RDP carboxylase for carbon dioxide fixation.

One function of the PEP carboxylase in the pericarp may be in the refixation of respired endosperm carbon dioxide. While 34% of gross ear photosynthesis is accounted for by photosynthesis in the wheat grain this figure is very much reduced (net photosynthesis) when correction is made for both dark and photorespiration (Evans and Rawson, 1970). The same authors, have, however put forward some evidence that the grains are capable of reassimilating most of their respired  $\text{CO}_2$ . PEP itself may originate by glycolysis from the starch of the pericarp amyloplasts. Both  $\alpha$ - and  $\beta$ -amylases are known to be present in this tissue during the developmental period (Banks *et al.*, 1972; Duffus and Rosie, 1973) and there is some evidence (unpublished) to suggest that starch in the pericarp is continually synthesised and digested during the early stages of grain development (Banks *et al.*, 1972; Duffus, 1969).

The high levels of PEP carboxylase, however, suggested a similarity to tissues such as maize or sugar cane in which PEP may arise from pyruvate by the action of the enzyme PEP synthetase. The evidence described here indicates that this might be a possibility. The presence of inorganic pyrophosphatase ensures the removal of the pyrophosphate released, thus favouring PEP synthesis.

Since there is no evidence to suggest that oxaloacetate or malic acid accumulate in the pericarp as is observed in the leaves of plants high in PEP carboxylase activity such as the Crassulaceae, those enzymes concerned with their further metabolism were investigated. Both malate NAD and NADP dehydrogenases which convert oxaloacetate to malate were present, activity being much greater with NAD than NADP. Malic enzyme catalyses the decarboxylation of malate to pyruvate. Thus there exists in the pericarp, at least in theory, a mechanism whereby PEP can be synthesised, carboxylated and finally regenerated. A net fixation of carbon dioxide is possible by this mechanism only if the carbon dioxide released by malic enzyme can be refixed—for example by RDP carboxylase in the Calvin cycle. Such a mechanism has been discussed by Slack and Hatch (1967). Certainly at least one other Calvin cycle enzyme, fructose 1,6 diphosphatase, has been shown to be present in this tissue.

The details of carbon metabolism in the pericarp can only be established by *in vitro* and *in vivo* studies of  $^{14}\text{CO}_2$  fixation. Whether or not

the tissue is capable of net fixation of carbon dioxide and its subsequent transport to the developing endosperm remains to be established.

The authors wish to thank the Scottish Plant Breeding Research Station, Pentlandsfield, East Lothian, for supplying the plant material.

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## PHOSPHORYLASE ACTIVITY IN RELATION TO STARCH SYNTHESIS IN DEVELOPING *HORDEUM DISTICHUM* GRAIN

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**Key Word Index**—*Hordeum distichum*; Gramineae; barley; endosperm; phosphorylase; isoenzymes; gel electrophoresis; starch synthesis.

**Abstract**—Activity, control and primer requirements of starch phosphorylase in developing barley endosperm were investigated. Phosphorylase was detected in endosperm extracts from 3 days after anthesis. Unprimed activity was predominant between 2 and 10 days after anthesis, when it constituted 70–80% of total activity, but this proportion declined rapidly as the grain developed. The existence of at least 2 isoenzymes was indicated by studies of pH dependence and phosphate inhibition, and was further supported by acrylamid



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### INTRODUCTION

It is generally considered that starch synthesis in plants is catalysed mainly by ADPG\*-starch transglucosylase (ADP glucose:  $\alpha$ -1,4-glucan  $\alpha$ -4 glucosyltransferase, E.C. 2.4.1.11., starch synthetase), and, to a lesser extent, by the related UDPG enzyme.<sup>1,2</sup> However, both of these enzymes require a short chain glucan primer. Leloir *et al.*<sup>3</sup> have shown that addition of maltosaccharides to a particulate starch synthetase system from developing *Phaseolus aureus* decreased the incorporation of labelled glucose into starch, longer chain maltosaccharides being preferentially synthesized. This suggests that transglucosylases are predominantly chain-lengthening enzymes. Thus the mechanism by which short chain glucans are initiated remains uncertain.

It has been reported<sup>4</sup> that phosphorylases ( $\alpha$ -1,4-glucan: orthophosphate glucosyltransferase, E.C. 2.4.1.1.) from certain plant systems are capable of synthesizing an amylose-like glucan in the absence of added primer. Tsai and Nelson<sup>5</sup> isolated from developing maize endosperm two phosphorylase isoenzymes, one of which could utilize maltose as a primer, and also synthesize starch in a 'primer-free' system. In destarched chloroplasts

\* Abbreviations: ADPG—ADP-glucose; cyclic AMP—3'5'-cyclic adenosine monophosphate; G1P—D-glucose-1-phosphate; G\*1P—D-[U-<sup>14</sup>C]-glucose-1-phosphate; G6P—D-glucose-6-phosphate; UDPG—UDP-glucose; UDPG\*—uridine-5'-diphospho-[U-<sup>14</sup>C]-glucose.

<sup>1</sup> DE FEKETE, M. A. R., LOLOIR, L. F. and CARDINI, C. E. (1960) *Nature* **187**, 918.

<sup>2</sup> RECONDO, E. and LOLOIR, L. F. (1961) *Biochem. Biophys. Res. Commun.* **6**, 85.

<sup>3</sup> LOLOIR, L. F., DE FEKETE, M. A. R. and CARDINI, C. E. (1961) *J. Biol. Chem.* **236**, 636

<sup>4</sup> SLABNIK, E. and FRYDMAN, R. (1969) *Biochem. Biophys. Res. Commun.* **38**, 709.

<sup>5</sup> TSAI, C. Y. and NELSON, O. E. (1968) *Plant Physiol.* **43**, 103.

synthesis of a glucan polymer from G1P in the absence of added primer, and its incorporation into starch by transglucosylase enzymes have been demonstrated by Bird.<sup>6</sup>

Phosphorylase activity and control have therefore been investigated both in crude and partially purified extracts from developing barley endosperm, with and without added glucan primers.

## RESULTS

### Phosphorylase Activity

Starch phosphorylase could be detected in barley endosperm extracts from as early as 3 days after anthesis (Fig. 1). Using soluble starch as a primer, maximum activity was attained around 22 days. Somewhat higher values, following the same developmental pattern, could be obtained with amylopectin, but soluble starch was used routinely because of its greater solubility. Unprimed activity was only slightly lower than total values during the first 10 days after anthesis, but remained considerably lower than total activity as development progressed. When unprimed activity, was expressed as a percentage of total activity, it became apparent that the highest proportion of unprimed activity occurred during the first

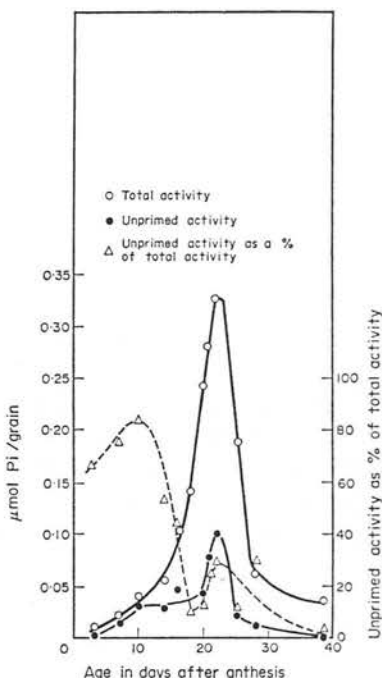


FIG. 1. PHOSPHORYLASE ACTIVITY IN SOLUBLE ENDOSPERM EXTRACTS DURING ENDOSPERM DEVELOPMENT.

Reaction mixtures contained: 15  $\mu$ mol Tris-maleate pH 6.2; 10  $\mu$ mol G1P; 0.2 ml soluble endosperm extract; 0.3 ml 2.5% soluble starch (for total activity). Mixtures were incubated at 25° for 2 hr and values corrected for controls without G1P and without endosperm extract.

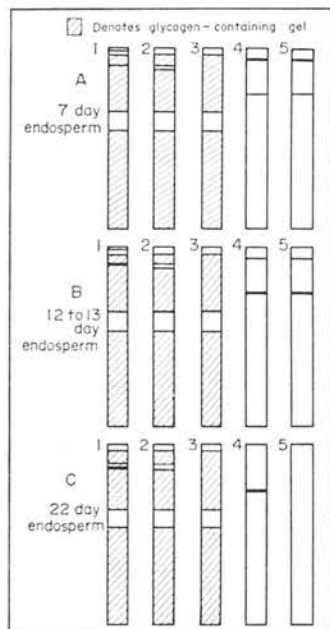


FIG. 2. PHOSPHORYLASE ACTIVITY AFTER ELECTROPHORESIS OF SOLUBLE ENDOSPERM EXTRACTS USING 7% POLYACRYLAMIDE GELS.

For conditions of electrophoresis, see text. Gels incubated overnight in: (1) 0.1 M citrate buffer, pH 5.0 + 0.025 M G1P; (2) 0.1 M citrate buffer, pH 5.0 + 0.1 M phosphate buffer pH 6.0; (3) 0.1 M citrate buffer; (4) 0.5% glycogen in citrate buffer + 0.025 M G1P; or 0.025 M G1P + 0.1 M citrate buffer.

<sup>6</sup> BIRD, I. F. (1969) Ph.D. Thesis, University of London.



10 days after anthesis, and declined during maturation. The increase in unprimed activity around 22 days (Fig. 1) was perhaps due to the accumulation in the endosperm extract of soluble oligosaccharides, sufficient to prime the phosphorylase reaction in the absence of added primer. This requires further investigation. Activity was chiefly located in the starchy endosperm, rather than in the aleurone layer. The enzyme appeared soluble, and traces of activity associated with the amyloplast fraction were readily removed by washing with buffer. There was no detectable increase in *free glucose* during the incubation indicating that the GIP was not being broken down by phosphatase activity.

Phosphorylase activity, measured in the direction of starch synthesis, was progressively inhibited by increasing concentrations of inorganic phosphate (Table 1). The extent of inhibition appeared dependent upon both the age of the endosperm and the presence or absence of primer. Primed activity was inhibited by increasing inorganic phosphate concentrations to a greater extent in 7- and 14-day extracts than in 22-day extracts. Conversely, unprimed activity was inhibited less in young grain than in older ones.

TABLE 1. EFFECT OF INORGANIC PHOSPHATE AND ADENOSINE NUCLEOTIDES ON PHOSPHORYLASE ACTIVITY

Addition (final concn) (M)		Activity as a % of activity in untreated extract*					
		Primed			Unprimed		
		Age in days after anthesis					
		7	14	22	7	14	22
Soluble endosperm extract	$2 \times 10^{-3}$ Pi†	92	96	85	91	81	71
	$2 \times 10^{-2}$ Pi	80	71	83	67	67	52
	$2 \times 10^{-1}$ Pi	16	28	64	17	2	0
	$10^{-4}$ cyclic AMP	—	90	—	—	63	—
	$10^{-4}$ AMP	—	47	—	—	68	—
	$10^{-4}$ ATP	—	40	—	—	26	—
Filtered homogenate	$70^{-2}$ cyclic AMP	100	—	—	75	—	—

\* Reaction conditions as for Fig. 1. Each result is the average of three determinations.

† Abbreviation: Pi, inorganic phosphate.

At a final concentration of  $10^{-4}$  M, cyclic AMP, AMP and ATP were inhibitory to both primed and unprimed phosphorylase in 14 day endosperm extracts. No significant effects could be induced by increasing the concentration of cyclic AMP to  $10^{-2}$  M (Table 1).

A fairly broad spectrum of pH dependence of both primed and unprimed activity was obtained with young grain, maximum activity shifting from pH 6 in 7-day endosperm extracts to pH 7 with 14-day extracts. With 22-day extracts a sharp peak of primed activity occurred around pH 7, while unprimed activity remained relatively unchanged between pH 6 and 7.

#### *Incorporation of Glucose into Starch*

Soluble extracts from young endosperm incorporated [ $^{14}$ C]-glucose from G\*IP into a water insoluble fraction which pelleted with carrier starch. In the presence of UDPG, glucose from G\*IP was incorporated by 2–3 day endosperm extracts (Table 2). In older grain, UDPG appeared to exert an inhibitory influence. The progressive incorporation of glucose with time was investigated using 5-day extracts. Addition of UDPG to the reaction



mixture had little effect during the first 10–12 hr of incubation, but a significant reduction in glucose incorporation became apparent as the incubation period was prolonged to 24 hr. Considerable incorporation, apparently unaffected by UDPG, was obtained with 16-day extracts.

TABLE 2. INCORPORATION OF [ $^{14}$ C]-GLUCOSE FROM G\*IP INTO STARCH, IN ABSENCE OF ADDED PRIMER

Glucosyl donor	Age in days after anthesis	cpm grain					
		3 hr		12 hr		24 hr	
		+	–	+	–	+	–
		UDPG	UDPG	UDPG	UDPG	UDPG	UDPG
1.0 $\mu$ Ci G*IP	2–3			74	0		
	3–4			754	11		
	5–6	200	250	350	250	1500	6900
	(all dissected immediately)						
0.5 $\mu$ Ci G*IP + 0.1 $\mu$ mol GIP	5–7 (dissected immediately)					1408	2042
	5–7 (left in H <sub>2</sub> O overnight)					2276	779
0.5 $\mu$ Ci G*IP + 0.1 $\mu$ mol GIP	16 (dissected immediately)					3072	3239

Reaction mixtures contained: 0.2 ml soluble endosperm extract; 15  $\mu$ mol Tris-maleate, pH 6.2; 1.0 or 0.5  $\mu$ Ci G\*IP; 0.1  $\mu$ mol GIP carrier where specified; 0.6  $\mu$ mol unlabelled UDPG where indicated. Incubation at 37°. All values corrected for background counts and for controls (see text).

Incorporation of [ $^{14}$ C]-glucose from UDPG\* into starch by transglucosylase activity was reduced when unlabelled GIP was included in the reaction mixture for both 12- and 24-hr incubations with 5–7 day soluble endosperm extracts (Table 3). No incorporation could be detected by 2–3 day extracts over 4, 12 or 24 hr.

In some cases barley ears 5–7 days after anthesis were allowed to stand in H<sub>2</sub>O overnight before endosperm extracts were prepared. It was then observed that increased incorporation of [ $^{14}$ C]-glucose into starch was obtained from either G\*IP or UDPG\* when both glucosyl donors were present (Tables 2 and 3).

Chromatography of endosperm extracts after incubation with either G\*IP or UDPG\* demonstrated the accumulation in the reaction mixture of a labelled product which remained immobile in the solvent system used. A series of labelled substances lying between the origin and the maltose reference spot could also be observed; these were especially pronounced in incubations containing both G\*IP + UDPG or GIP + UDPG\*. These spots were no longer visible, and the proportion of immobile material was significantly reduced, when the reaction mixture was incubated with  $\beta$ -amylase before chromatography (Table 4). A concomitant increase in labelled maltose was observed. The apparent larger amount of activity in the maltose after treatment is due to its release from oligosaccharides

TABLE 3. INCORPORATION OF [ $^{14}$ C]-GLUCOSE FROM UDPG\* INTO STARCH IN ABSENCE OF ADDED PRIMER, BY 5-7 DAY ENDOSPERM EXTRACTS

Length of incubation (hr)	Fraction	Glucosyl donor	cpm/grain	
			+ G1P	- G1P
12	Endosperm homogenate	0.1 $\mu$ Ci UDPG	66	119
24	Soluble endosperm extract	0.1 $\mu$ Ci UDPG	41	268
24	Soluble endosperm extract	0.05 $\mu$ Ci UDPG* + 0.3 $\mu$ mol UDPG	18	51
12	Soluble endosperm extract left in H <sub>2</sub> O overnight	0.1 $\mu$ Ci UDPG	184	82

Reaction conditions as for table 2, with 0.1 or 0.5  $\mu$ Ci UDPG\* as labelled glucosyl donor; 0.3  $\mu$ mol UDPG carrier; 0.1  $\mu$ mol unlabelled G1P where indicated. All values corrected for background and controls (see text).

as well as the non-mobile polymer. The immobile material is thus identified as a long chain  $\alpha$ -1,4 glucan, and the slow moving components as maltosaccharides. Glucans synthesized from G\*1P alone gave rise to a much higher proportion of maltose than material synthesized from UDPG\* alone. Incubations containing both G1P and UDPG produced approximately equivalent amounts of free maltose.

TABLE 4. RADIOACTIVITY OF SPOTS ELUTED FROM CHROMATOGRAM SHOWING EFFECT OF  $\beta$ -AMYLASE ON NEWLY SYNTHESISED GLUCAN

Glucosyl donors	$\beta$ -Amylase treatment	cpm/grain	
		At origin	At maltose spot
G*1P + UDPG	Before	3440	498
	After	1747	8313
G1P + UDPG*	Before	635	482
	After	194	1597
G*1P	Before	2411	384
	After	1810	11 858
UDPG*	Before	1281	388
	After	1043	646

Reaction mixtures contained: 0.5  $\mu$ Ci G\*1P + 0.1  $\mu$ mol G1P carrier; 0.6  $\mu$ mol UDPG where indicated; or 0.1  $\mu$ Ci UDPG\*; 0.1  $\mu$ mol G1P where indicated. Incubated at 37° for 24 hr, then treated with 0.1 ml  $\beta$ -amylase (1 mg/1 ml) for 1.5 hr at 23°. All values corrected for background radiation.

#### *Pretreatment of Endosperm Extract with Amylases*

Both primed and unprimed phosphorylase activity increased considerably after incubation of 7- and 15-day endosperm extracts with glucoamylase (Table 5). The extent of increase was reduced by dialysis but remained significant. No significant change in activity was noted after preincubation with  $\beta$ -amylase. A similar activation occurred with 50% saturated

(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractions pre-incubated with glucoamylase. In these fractions unprimed activity, although present initially, was abolished during incubation with boiled glucoamylase, suggesting an increased instability of the enzyme after (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> treatment.

Incorporation of [<sup>14</sup>C]-glucose from G\*1P into starch was similarly increased by glucoamylase pretreatment, the activation being more pronounced in the absence of UDPG (Table 5).

TABLE 5. PHOSPHORYLASE ACTIVITY IN SOLUBLE ENDOSPERM EXTRACTS PRETREATED WITH GLUCOAMYLASE

Enzyme source	Glucosyl donor	Treatment	$\mu\text{mol Pi}/10^3 \text{ grain} \pm \text{s.d.}$			
			Primed		Unprimed	
			Not dialysed	After dialysed	Not dialysed	After dialysed
7-day soluble endosperm extract	G1P	+ Glucoamylase Control	57 $\pm$ 3 30 $\pm$ 7	28 $\pm$ 3 10 $\pm$ 1	50 $\pm$ 3 15 $\pm$ 1	28 $\pm$ 8 6 $\pm$ 2
15-day soluble endosperm extract	G1P	+ Glucoamylase Control	80 $\pm$ 10 15 $\pm$ 4	45 $\pm$ 9 15 $\pm$ 5	75 $\pm$ 3 16 $\pm$ 4	33 $\pm$ 2 9 $\pm$ 3
50% sat. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ppt. of 7-day extract	G1P	+ Glucoamylase Control	$\mu\text{mol Pi}/\text{mg Protein}$			
			7.94 0.88	4.72 0.55	6.83 0	4.44 0
			Incorporation of [ <sup>14</sup> C]-glucose into starch without primer cpm/grain			
			+UDPG		-UDPG	
3-day soluble endosperm extract	0.25 $\mu\text{Ci}$ G*1P + 0.5 $\mu\text{mol}$ G1P	+ Glucoamylase Control	333 130		367 —	
5-day soluble endosperm extract	0.25 $\mu\text{Ci}$ G*1P + 0.5 $\mu\text{mol}$ G1P	+ Glucoamylase untreated	863 257		3650 293	
5-day soluble endosperm extract	0.5 $\mu\text{Ci}$ G*1P + 0.1 $\mu\text{mol}$ G1P	+ Glucoamylase untreated	2583 319		6359 432	

Reaction conditions as for Fig. 1 and Table 3. Endosperm extract (2 ml) pretreated with 0.2 ml glucoamylase (50 mg/ml) for 2 hr at 30°. (Control, boiled glucoamylase.)

### Electrophoresis

After fractionation on glycogen-containing gels 7- and 12–13-day soluble endosperm extracts showed two bands of synthetic phosphorylase activity, (1)  $R_f$  0.01, and (2)  $R_f$  0.13 (Fig. 2 A1, B1). Component (1) was more pronounced in 7-day extracts while (2) predominated in 12–13 day extracts. With 22-day extracts only (2) was visible, split into 2 bands,  $R_f$  0.11 and 0.13 (Fig. 2, C1).

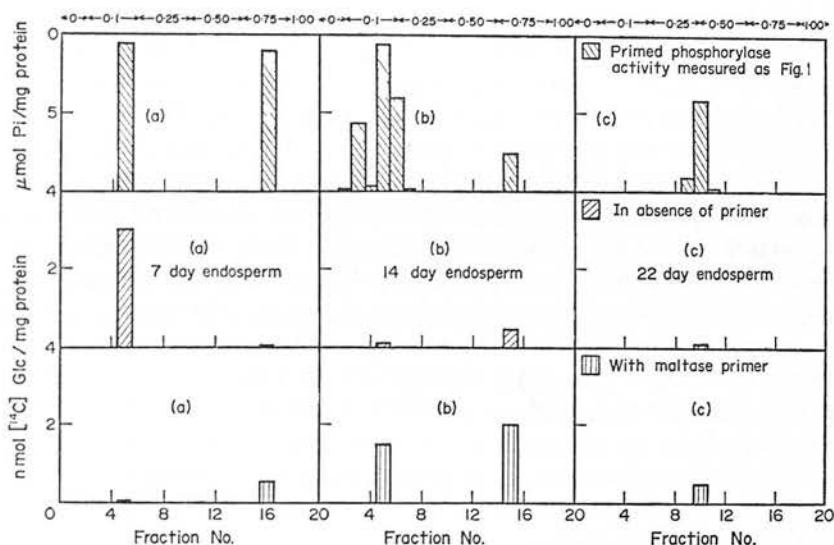
Substitution of inorganic phosphate for G1P in the incubation mixture resulted in colourless degradative bands of the same  $R_f$ s (Fig. 2, A2, B2, C2). Colourless amylase bands ( $R_f$  0.028 and 0.42), which remained unchanged from 7 to 22 days, were distinguishable

from phosphorylase bands by omitting inorganic phosphate and G1P from the incubation mixture, in which case only amylase bands were visible (Fig. 2, A3, B3, C3).

Glycogen-free gels incubated in glycogen + G1P showed similar zoning, except that fractions migrated slightly faster and were less well defined (Fig. 2, A4, B4, C4). Dark bands occurred at  $R_f$  0.045 and 0.23 with 7- and 12–13-day extracts, and at  $R_f$  0.23 with 22-day extracts. If glycogen was completely omitted synthetic bands could still be distinguished, (1) being more prominent in 7-day extracts, and (2) in 12–13 day extracts. No bands were distinguishable with 22-day extracts (Fig. 2, A5, B5, C5).

### DEAE-cellulose Chromatography

Extracts from 4-day endosperm could be resolved into 2 peaks of phosphorylase activity, (1) (fraction 4), and (2) (fraction 16) (Fig. 3). Only (1) could synthesize an insoluble glucan polymer from G\*1P in the absence of added primer, although (2) showed slight activity with maltose. Twelve-day extracts gave 3 active peaks, 2 of which were very close and probably corresponded to (1), while the third peak corresponded to phosphorylase (2). Incorporation of glucose into an insoluble glucan polymer was negligible in all fractions (except fraction 15) in the absence of added primer, but was increased by the addition of maltose.



3. FRACTIONATION OF BARLEY ENDOSPERM PHOSPHORYLASE BY DEAE-CELLULOSE CHROMATOGRAPHY (for conditions of chromatography see text). Incorporation of [ $^{14}$ C]-glucose from G\*1P estimated as for Table 2—in absence of primer with maltose primer.

Only one active fraction was obtained from 22-day extracts: this was eluted in fractions 9 and 10, and did not appear to correspond to either (1) or (2) of 7- and 12-day extracts. This fraction was almost inactive in the absence of added primer, and only slightly activated by addition of maltose.

No carbohydrate could be detected in any fractions. The assay method used<sup>7</sup> could detect minimum glucose concentrations of 1  $\mu$ g/ml.

<sup>7</sup> MORRIS, D. L. (1948) *Science* **107**, 254.

## DISCUSSION

In barley endosperm, soluble phosphorylase could be detected several days before particulate starch synthetase, although periods of maximum activity of both enzymes coincided.<sup>8</sup> Unprimed activity was prominent between 2 and 10 days after anthesis, when it constituted 70–80% of total activity, but this proportion declined rapidly as starch synthetase increased.

The variation of inhibition by inorganic phosphate with both the age of the grain and the presence of primer suggested that two or more phosphorylase isoenzymes could be present in the endosperm, having different susceptibilities to inorganic phosphate, and different primer requirements. Changes in the relative concentrations of such isoenzymes may indicate a means of controlling the rate and direction of phosphorylase activity *in vivo*.

Indications of enzyme multiplicity could also be drawn from pH dependence measurements. The broad spectrum of activity with 7- and 14-day extracts suggested a mixture of protein, whereas the sharper activity maximum of 22-day extracts implied a single protein. The negative effects of adenosine nucleotides on enzyme activity supported previous evidence<sup>5,9</sup> that starch phosphorylase in higher plants does not consist of subunits activated by adenosine nucleotides in the same way as glycogen phosphorylase in animal systems.

Since it is probable that glucans synthesised by phosphorylase, either *de novo* or using small maltosaccharide precursors, may be available as primers for transglucosylase activity, then incubations with G1P and UDPG (or ADPG) should show incorporation of glucose from both glucosyl donors.<sup>6</sup> Incorporation from G\*1P was accelerated by UDPG with 2–3 day endosperm, but inhibited with 5–7 day endosperm. ADPG and UDPG have been shown to inhibit starch and glycogen phosphorylase.<sup>10,11</sup> The lack of inhibition with 2–3, and 16-day extracts suggests that the effect may be due to competition between the enzymes for available primer. Assuming that 2–3 day endosperms contain no endogenous primer, synthesis of starch by phosphorylase will be extremely slow, and thus addition of UDPG (allowing elongation of labelled glucans) will significantly increase measurable incorporation from G\*1P. By 5–7 days, limited quantities of endogenous primers in the endosperm will cause competition between the enzymes, but by 16 days, primer will no longer be limiting. Similarly incorporation from UDPG\* will be reduced by the addition of G1P. Enhanced incorporation from G1P and UDPG together was obtained by Bird<sup>6</sup> using destarched chloroplasts, which presumably contained no endogenous primer, and corresponded to the situation with 2–3 day endosperm. Similar results could be induced by leaving barley ears in water overnight before preparing endosperm extracts, so that endogenous primers were used up in respiration.

The higher susceptibility of starch synthesised from G\*1P alone to  $\beta$ -amylase attack indicates that phosphorylase synthesises *de novo* only straight chain glucans, while both amylopectin and amylose result from transglucosylase activity.<sup>10,12,13</sup>

Since starch phosphorylase can utilize shorter chain primers than muscle phosphorylase,<sup>14</sup> unprimed activity may be due to priming by endogenous maltosaccharides, or by carbohydrate contaminants of substrates. However, glucose was incorporated by soluble

<sup>8</sup> BAXTER, E. D. and DUFFUS, C. M. (1971) *Phytochemistry* **10**, 2641.

<sup>9</sup> GREEN, D. E. and STUMPF, P. K. (1942) *J. Biol. Chem.* **142**, 355.

<sup>10</sup> DE FEKETE, M. A. R. (1969) *Planta* **87**, 311.

<sup>11</sup> MADSEN, N. B. (1961) *Biochem. Biophys. Res. Commun.* **6**, 310.

<sup>12</sup> HANES, C. S. (1940) *Proc. Roy. Soc. (London)* **128 B**, 421.

<sup>13</sup> OZBUN, J. L., HAWKER, J. S. and PREISS, J. (1971) *Biochem. Biophys. Res. Commun.* **43**, 631.

<sup>14</sup> CORI, G. T., SWANSON, M. A. and CORI, C. F. (1945) *Federation Proc.* **4**, 234

endosperm extracts using only G\*1P (purified by chromatography and therefore free from low MW contaminants) as substrate. Furthermore, Bird<sup>6</sup> found comparable rates of unprimed activity using purified or unpurified G1P.<sup>15</sup> Contamination of G1P does not, therefore appear significant.

Contamination by soluble endogenous primers remains probable. The increase in primed activity after treatment of crude extracts with glucoamylase is probably due to breakdown of the starch primer, giving rise to more short-chain glucans. Increase in unprimed activity suggests that the crude extract contained a limited amount of maltosaccharides which could be broken down by glucoamylase, yielding a larger number of suitable primer chains. Shorter chains would be lost on dialysis, reducing the extent of activation, but longer chains, or possibly protein-bound glucans, would be retained. Persistence of glucoamylase-induced activation in 50% satd  $(\text{NH}_4)_2\text{SO}_4$  fractions supports a hypothesis of protein-bound glucan primers.

Purification of endosperm extracts by electrophoresis and chromatography indicated the presence of multiple forms of phosphorylase. In each procedure two isoenzymes could be isolated from endosperms up to 15 days after anthesis. Unprimed activity was associated exclusively with phosphorylase (1) in very young endosperm, but as development proceeded this potentiality was acquired by phosphorylase (2). As (2) accumulated, phosphorylase (1) disappeared. By 22 days after anthesis only one isoenzyme, which required a glucan primer, persisted. The loss of unprimed activity may reflect modifications of the protein without alteration of electrophoretic mobility, since the isoenzyme detected in 22 day endosperm did not correspond to either phosphorylase (1) or (2) of younger endosperm, as separated by DEAE-cellulose chromatography.

Two phosphorylase isoenzymes, separable by acrylamide gel electrophoresis, have been identified<sup>10</sup> in spinach leaf extracts and immature *Vicia faba* cotyledons. Gerbrandy and Verleur,<sup>16</sup> who separated several phosphorylase isoenzymes from potatoes, found that different isoenzymes appeared in young and mature tubers, and suggested that certain isoenzymes were active mainly in the direction of synthesis, whilst others were concerned with starch degradation. No unprimed activity was detected<sup>5</sup> in maize endosperms fractionated by DEAE-cellulose until 12 days after anthesis but since incubation periods were only 30–45 min duration it is quite possible that low levels of unprimed activity in very young endosperm were not detected.

Maintenance of unprimed activity in young endosperm throughout purification procedures reduces the possibility of its being attributable entirely to contamination by endogenous maltosaccharides, and supports the hypothesis of a protein-bound glucan primer, possible attached to the phosphorylase enzyme. Concentrations of such a bound primer could well be too low to be detected in the eluate by the method used.<sup>7</sup> For example, Fukui and Kamogawa<sup>17</sup> found 0.2–0.6 glucose residues/mol enzyme protein in a crystalline preparation of potato phosphorylase. Removal of this carbohydrate by glucoamylase abolished unprimed activity. Fredrick<sup>18</sup> obtained similar results with algal phosphorylase, and suggested that the isoenzymes were glycoproteins.

The results of the present investigation are consistent with the appearance in the barley endosperm soon after anthesis of two phosphorylase isoenzymes, possibly both glycoproteins. Phosphorylase (1) appears capable of unprimed activity, and may be associated

<sup>15</sup> KAMOGAWA, A., FUKUI, T. and NIKUNI, Z. (1968) *J. Biochem.* **63**, 361.

<sup>16</sup> GERBRANDY, S. J. and VERLEUR, J. D. (1971) *Phytochemistry* **10**, 261.

<sup>17</sup> FUKUI, T. and KAMOGAWA, A. (1969) *J. Jap. Soc. Starch Sci.* **17**, 117.

<sup>18</sup> FREDRICK, J. F. (1971) *Physiol. Plant.* **25**, 32.



with the initiation of  $\alpha$ -1,2-glucans, which then serve as primers for starch synthetase. This isoenzyme disappears after 13–15 days. Phosphorylase (2) is capable of some unprimed activity but undergoes modification between 15 and 20 days after anthesis (possibly by removal of the carbohydrate moiety), resulting in loss of unprimed activity.

### EXPERIMENTAL

**Materials.** Glucoamylase (E.C. 3.2.1.3. specific activity 2700 mg Glc/min/g at 55°, pH 4.5) and  $\beta$ -amylase (E.C. 3.2.1.2. specific activity 15 mg maltose/min/mg at 20°, pH 4.8) and most biochemicals and substrates including corn endosperm amylopectin and oyster glycogen were obtained from Sigma Chemical Co. (St. Louis, Mo. U.S.A.). Soluble starch was obtained from British Drug Houses (London), and radioactively labelled substrates from The Radiochemical Centre, (Amersham, Bucks.). The two row barley *Hordeum distichum* (L) Lam. cv. Maris Baldric, was used throughout, as described previously.<sup>8</sup> Soluble endosperm extracts were prepared by suspending the endosperm (including the aleurone layer) in Tris-maleate buffer (0.1 M, pH 6.2) and homogenizing by hand in an all-glass homogenizer. The filtered homogenate was centrifuged for 10 min at 10 000 g, 4°. The supernatant solution constituted the soluble endosperm extract. Pelleted material, which upon examination under the microscope ( $\times 1000$ ) appeared to consist mainly of amyloplasts, was washed and resuspended in buffer, and constituted the insoluble endosperm extract. In older grain (20 days after anthesis) the aleurone layer could be scraped off the endosperm, and a soluble extract prepared as for the endosperm. In some cases the soluble endosperm extract was treated for 2 hr at 30° with glucoamylase (50 mg/ml soln) or  $\beta$ -amylase (2 mg/2 ml soln).

**Phosphorylase assay.** This was measured by the release of inorganic phosphate from G1P<sup>19</sup> in Tris-maleate buffer pH 6.2. Soluble starch or amylopectin was used as a primer. Inorganic phosphate was estimated according to Lowry and Lopez<sup>20</sup> using 2% ascorbic acid. All values were corrected for endogenous inorganic phosphate and non-enzymatic breakdown of G1P. Samples were also tested for any increase in free glucose after incubation. Total activity detected in the presence of added primer is referred to as 'primed activity', and activity with no added primer as 'unprimed activity'.

**Incorporation of [<sup>14</sup>C]-glucose into starch.** After incubation of endosperm extracts with G\*1P (specific activity 277 mCi/mmol) or UDPG\* (233 mCi/mmol) at 37° for periods of up to 24 hr, solid starch was added as a carrier and insoluble material collected by centrifugation (10 min, 4°, 13 000 g), washed several times to remove unreacted material, and the radioactivity estimated in a Beckmann liquid scintillation system after resuspending the pellet in 0.5 ml boiling H<sub>2</sub>O. Values were corrected for background radiation and controls with endosperm extract. Counting efficiency for <sup>14</sup>C was about 25%. Labelled products were separated by descending PC for 20 hr using a PrOH-EtOAc-H<sub>2</sub>O (6:1:3) system,<sup>21</sup> and visualized by exposure to an X-ray film. In some cases product mixtures were treated with  $\beta$ -amylase (1 mg/ml) for 1.5 hr at 23° before chromatography.

**Electrophoresis.** This was carried out according to Rainer-Maurer<sup>22</sup> using gel system 1 with 7% polyacrylamide gels. Tris-glycine buffer, 0.1 M, pH 8.3, was used as reservoir buffer. A 0.4% soln of glycogen was polymerized with some gels to serve as a primer. Current (3 mA/tube) was passed for about 2 hr at room temp., using bromophenol blue as the front indicator. Gels were incubated overnight in a solution containing 3 ml each of 0.025 M G1P and 0.1 M citrate buffer, pH 5.0. With primer-free gels, glycogen was sometimes added to the incubation mixture. Gels were then stained in I<sub>2</sub>-KI soln.

**DEAE-cellulose chromatography.** That fraction of the soluble endosperm extract precipitating at 50% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation was collected by centrifugation (20 min at 34 000 g, 4°), suspended in Tris-maleate buffer (0.01 M, pH 7.0), dialysed against the same buffer, and a 1-ml sample applied to a DEAE-cellulose column (1  $\times$  20 cm) previously equilibrated with the same buffer.<sup>5</sup> The sample was eluted in a total vol of 110 ml by a linear discontinuous concentration gradient, from 0 to 1 M NaCl in Tris-maleate buffer, pH 7.0. 5.0 ml fractions were collected, dialysed overnight, and assayed for (a) phosphorylase activity, (b) incorporation of glucose into starch, (c) protein<sup>23</sup> and (d) carbohydrate content.<sup>7</sup>

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## BIOCHEMICAL CHANGES DURING EMBRYOGENY IN *HORDEUM DISTICHUM*

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**Key Word Index**—*Hordeum distichum*; Gramineae; barley; embryo; nucleic acids; sucrose synthetase; invertase.

**Abstract**—Changes in fr. and dry wt, soluble reducing sugars, protein, total carbohydrate, DNA, RNA, sucrose synthetase activity and invertase activity were recorded for the developing embryo of *Hordeum distichum* var Julia over the period 18–60 days after anthesis. Fresh wt increased until 45 days whereupon rapid dehydration commenced. Reducing sugar concentration remained low throughout development but total carbohydrate and protein accumulated rapidly over the initial period to reach maximum values at around 50 days. DNA concentration remained relatively constant throughout the middle and later stages of development, but RNA, on the other hand, increased rapidly to reach a maximum value at maturity. Sucrose synthetase (assayed in the direction of sucrose cleavage) was considerably more active with UDP than ADP and reached a maximum value around 35 days after anthesis. When assayed in the direction of sucrose synthesis the peak of activity was slightly later in development and doubled in value. Invertase activity was appreciable and was still present at maturity.

### INTRODUCTION

The morphological changes accompanying the initial stages of differentiation during embryogenesis in higher plants have been well described [1]. Little is known, however, of the associated biochemical changes. Jennings and Morton [2] studied the changes in grain composition with maturation. Nitrogen content of the embryos increased steadily from 18 days after anthesis. At maturity the embryo, which constituted *ca* 3% of total grain weight, contained about 7% of total grain N<sub>2</sub>. The same workers [3] have shown that total phosphorus showed a similar steady increase and that at maturity 14% of total grain phosphorus was present in the embryo.

Walbot *et al.* [4] in a recent study have described the developmental interaction between the suspensor and the organogenetic part of the embryo in terms of RNA metabolism. They showed that synthetic activity of the suspensor was highest early in development and then declined, whereas synthetic activity of the organogenetic part increased throughout development. The fine structure in cells of mature pea and wheat embryos has been examined by Setterfield *et al.* [5]. The nuclei of all cells were similar, showing nuclear membranes, chromosomes and prominent nucleoli.

Other structures present included amyloplasts, extensively developed endoplasmic reticulum, mitochondria and large numbers of ribonucleoprotein bodies—presumably ribosomes. The mature wheat scutellum has also recently been examined [6]. In this case nuclear chromatin was strongly aggregated and the endoplasmic reticulum, while present, was not abundant. Intact mitochondria and plastids were also visible. The fine structure of the developing embryo was not investigated.

Thus although RNA metabolism has been investigated in embryogenesis, the overall mechanism controlling embryo development has not. The present work attempts to characterize the broad features of embryogenesis by describing some of the accompanying biochemical changes. The results will be correlated where possible, with those already reported [7] for the developing seed and its associated tissues.

### RESULTS

#### *Overall biochemical changes in the embryo during development*

The changes in fr. and dry wt of the embryo are shown in Fig. 1. Fresh wt, after an initial lag phase, increased rapidly from 25 days after anthesis,

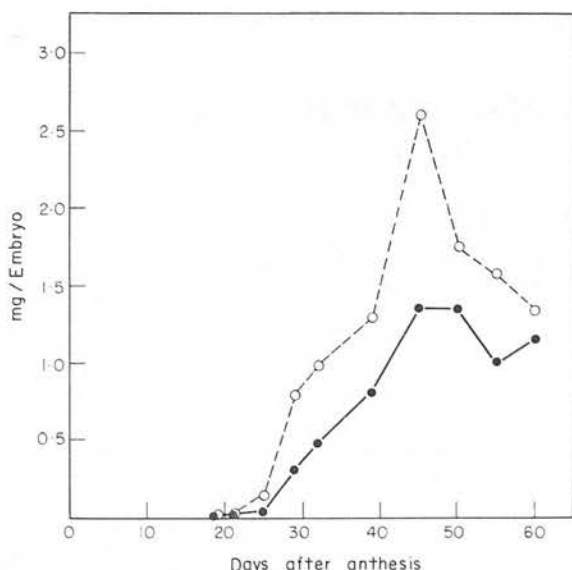


Fig. 1. Changes in DNA and RNA content of developing embryos. ●—● DNA; ○—○ RNA.  
CAPTION FOR FIGURE 3.

reaching a maximum value around 45 days and declining markedly thereafter. Dry wt also remained low until 25 days, then rose steadily to a maximum value at 39 days and did not fall much below this value with increasing maturity. Thus dehydration commences at 45 days and is almost complete by 60 days.

The variation during embryo development of total carbohydrate, reducing sugars and protein is given in Fig. 2. The level of total carbohydrate is initially low, but appreciably higher than that of reducing sugars at the same age. Rapid accumulation occurs between 18 and 45 days when a maximum figure is attained; levels then decrease slightly. The graph for protein concentration follows a similar pattern rising sharply over the initial period to produce a maximum value at around 50 days. A marked decrease is seen over the final stage of maturation. Levels of reducing sugars remain low throughout, with a gradual increase around 40 days to a broad maximum and a slow decrease thereafter.

After an initial slow increase in concentration, DNA levels remained relatively constant throughout the middle and later stages of development (Fig. 3). RNA concentration, on the other hand, increased rapidly throughout development to reach a maximum value only at maturity.

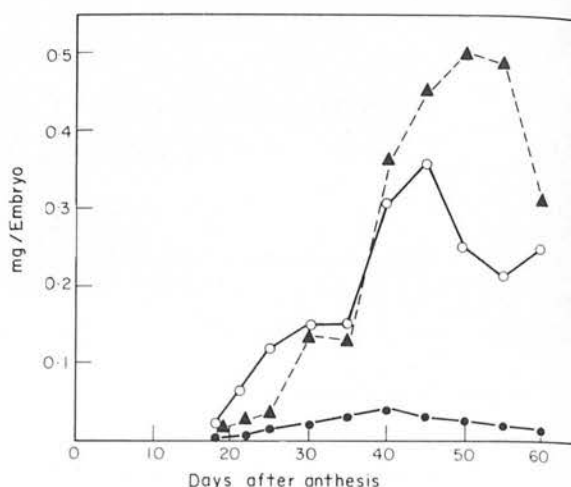


Fig. 2. Changes in reducing sugars, total carbohydrate and protein of developing embryos. ●—● reducing sugars; ○—○ total carbohydrate; ▲—▲ protein.

#### *Changes in the enzymes of carbohydrate metabolism during embryogenesis*

The sucrose synthetase (sucrose cleavage) in the embryos was considerably more active with UDP than with ADP (Fig. 4). Activity with UDP could be detected in 21 day embryos and increased very rapidly to a maximum value around 35 days. With ADP, activity was not detectable until 25 days and after reaching a maximum value around 30 days,

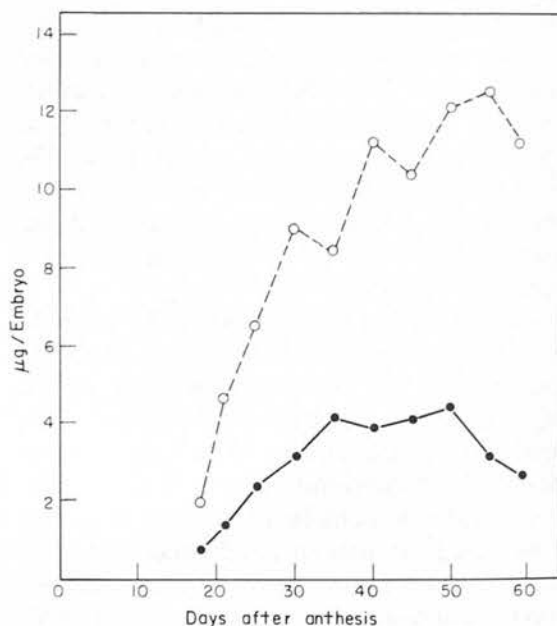


Fig. 3. Changes in fr. wt and dry wt of developing embryos. ○—○ fr. wt; ●—● dry wt.  
CAPTION FOR FIGURE 1.

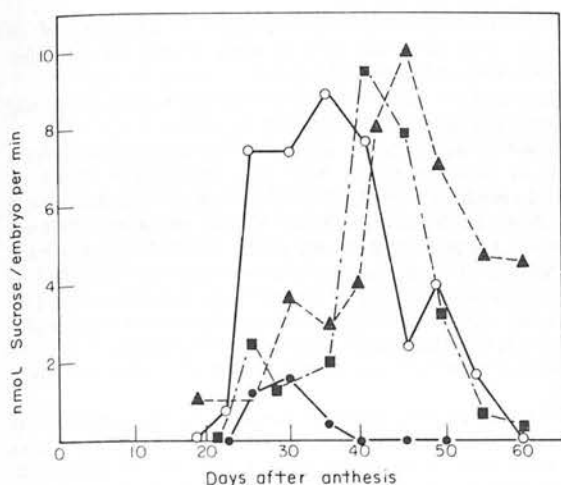


Fig. 4. Changes in UDP and ADP dependent sucrose synthetase activity (sucrose cleavage) and invertase activity of developing embryos. ○—○ UDP dependent sucrose synthetase; ●—● ADP dependent sucrose synthetase; ▲—▲ invertase; ■—■ sucrose synthetase activity (sucrose synthesis).

fell rapidly to zero at around 39 days. Activity with UDP fell to zero only at 60 days after anthesis. Invertase activity was always measurable in embryos. After an initially slow increase, enzyme activity increased rapidly to reach a maximum value at 45 days. Although activity decreased thereafter appreciable levels were still present at maturity.

Sucrose synthetase (sucrose synthesis) had a similar developmental pattern (Fig. 4) to UDP dependent sucrose synthetase (sucrose cleavage). The peak of activity was slightly later in development and almost doubled in value. The initial rate of increase was, however, much less than that of the cleavage enzyme.

The results for sucrose phosphate synthetase (sucrose synthesis) are not reported since: (a) at every age examined, activity with fructose-6-phosphate was much less than with fructose and (b) hydrolysis of fructose-6-phosphate (as measured by release of inorganic phosphate in the absence of added UDPG) was considerable and made it impossible to conclude that activity was due to fructose-6-phosphate rather than to fructose itself.

#### DISCUSSION

It is interesting to note that while the embryo initially grew fairly slowly, in terms of increase in fr. and dry wt, carbohydrate, protein, enzyme activity

and RNA concentration increased very rapidly over this period. Indeed, a major part of the young embryo must be composed of carbohydrate and, to a lesser extent, of protein. As development proceeded, however, the rate of protein accumulation exceeded that of carbohydrate. Final concentrations were similar.

These results contrast strongly with those described [7] for the developing barley endosperm, in which protein concentration varied little and constituted less than 20% of the mature tissue. Presumably the increase in protein levels is associated with the synthesis of enzyme protein since it parallels the increases in activity of most of the enzymes investigated.

While the reducing sugar levels remain low throughout embryogenesis carbohydrate synthesis and accumulation is rapid. The low levels thus do not reflect their probably high turnover rate. It is likely that, while the results do not show initially high levels of reducing sugar, embryo carbohydrate is synthesized from a precursor pool of reducing sugars [7]. Some of this carbohydrate is likely to be starch [20] and certainly our observations with the light microscope, of sections stained with  $I_2/KI$ , suggest that the barley embryo contains many small amyloplasts. The wheat [6] and barley [21] scutella apparently contain no amyloplasts and presumably, therefore, no starch.

The pattern of RNA accumulation follows very closely that described [22] for embryos of *Phaseolus vulgaris* L. except that synthesis tails off only at the very last stages of maturation. Thus protein synthesis, which also continues to maturity, is probably mediated by newly synthesized RNA.

Of particular interest was the observation that the DNA concentration remained relatively constant per embryo throughout the middle and later stages of development. The significance of these results in relation to cell division and differentiation will not be clear until more is known of the DNA concentration per cell at different stages of development.

The developmental pattern of the enzymes involved in sucrose cleavage was very similar to that recorded previously for endosperm [7]. Thus the increase in enzyme activity immediately preceded gains in dry wt, a major part of which was due to carbohydrate. Sucrose synthetase (sucrose cleavage) was considerably more active with UDP

than with ADP. It is therefore likely that the UDP enzyme is involved preferentially in the utilization of translocated sucrose. However, invertase was present at 18 days while neither ADP nor UDP dependent sucrose synthetase (sucrose cleavage) activity was detectable. It is possible that in the very early stages some sucrose is cleaved by invertase during entry and diffuses as monosaccharides to the embryo cells. The resynthesis of sucrose from monosaccharides, derived either from invertase or the cleavage enzymes, is then possible utilizing UDPG dependent sucrose synthetase (sucrose cleavage)—an enzyme shown here to be twice as active as the ADP dependent cleavage enzyme at its maximum. A similar mechanism was suggested by Shannon [23] for developing maize endosperm. The overall mechanism of carbohydrate synthesis in the embryo cannot however be established until the sites of action of enzymes such as invertase are determined. Edelman *et al.* [24] have shown that in germinating cereal seeds invertase activity is confined to the root and shoot and very little is found in the scutellum. On the other hand Palmer [21] has shown that in barley after 2 hr germination, invertase activity was measurable in root, shoot and scutellum. Certainly invertase was noteworthy in that its activity did not fall to zero at maturity. It may be that, like  $\beta$ -amylase [25], it is reactivated from a latent form in germination.

Thus, while some of the broad features accompanying embryogenesis in barley have been described, the details remain to be established. In particular the origin of the nutrients supplied to the developing embryo is subject to some speculation. It is generally supposed that the embryo requires the endosperm for development and presumably some, at least, of the carbon and nitrogen required is derived from the immature endosperm. These, and related problems are currently under investigation in this laboratory.

#### EXPERIMENTAL

*Plant material.* The two row barley *Hordeum distichum* (L.) Lam. cv. Julia, was used throughout. Conditions of growth and methods used to determine the date of anthesis were as described by Merritt and Walker [8].

*Embryo extracts.* The outer layers of glumes, paleae and pericarp were first taken off the intact grains. The embryos, together with the scutellum were then removed by hand and analysed immediately. The term "embryo" thus refers to the scutellum

together with the organogenetic part. A minimum of 10 embryos from the grain aged between 18 and 60 days after anthesis was used for each analysis.

*Fresh and dry weights.* Fresh wts were first determined and the embryos then dried to constant wt at 80°.

*Soluble reducing sugars.* Embryos were homogenized vigorously in 1.0 ml of 0.01 M NaF. The homogenate (which included a further 0.5 ml H<sub>2</sub>O used for washing the homogenizer) was then centrifuged at 3000 g for 10 min. The supernatant was removed, the pellet re-extracted with a further 0.5 ml H<sub>2</sub>O and the suspension centrifuged as before. A suitable volume, i.e. 0.1–1.0 ml depending on size of the embryos, was then assayed directly for soluble reducing sugars by the Somogyi–Nelson procedure [9, 10]. Deproteinization was not necessary.

*Total carbohydrate* was measured using the anthrone reagent [11].

*DNA, RNA and protein* were measured in intact embryos by the method described by Rozijn and Tonino [12]. The values for RNA were additionally verified after sodium lauryl sulphate–phenol extraction as described by Stern [13].

*Invertase.* Embryos were homogenized in 1.0 ml of 0.2 M acetate buffer pH 4.8 and the suspension assayed for invertase at pH 4.8 as described by Tsai *et al.* [14]. The reducing sugars released were measured as described above.

*Sucrose synthetase* (sucrose cleavage). UDP and ADP dependent sucrose synthetase was assayed in the 3000 g for 10 min supernatant as described by Pressey [15]. The incubation time was 10 min. Controls were run in the absence of UDP (ADP) to correct for invertase activity. The fructose released was measured by the Somogyi–Nelson procedure.

*Sucrose synthetase* (sucrose synthesis). The reaction mixture contained 20  $\mu$ mol HEPES buffer pH 7.4, 2  $\mu$ mol fructose, 0.5  $\mu$ mol UDPG and 0.05 ml enzyme in a total vol. of 0.25 ml. After incubation for 10 min at 37° the sucrose released was measured by the method of Roe [16] as modified by Cardini *et al.* [17]. Controls were run simultaneously in which either UDPG or fructose were added after incubation.

*Sucrose phosphate synthetase* (Sucrose synthesis). This was assayed as described for sucrose synthesis above except that fructose was replaced by fructose-6-phosphate and Tris-HCl buffer pH 6.4 replaced the HEPES buffer [18]. Inorganic phosphate released by fructose-6-phosphate under these conditions but in the absence of added UDPG was measured by the method of Allen [19].

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## Purification and Fractionation of Potato Amyloplasts

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A method is described for the isolation and purification of amyloplasts from mature potato tubers. The low-viscosity, high-density materials Urografin and Ludox TM were used in density gradients to obtain an additional fractionation on the grounds of size. Two main populations were identified. The properties of these materials and the purity of the amyloplast fractions are discussed.

No method has yet been devised for the satisfactory purification and fractionation of amyloplasts. Pottinger and Oliver (1) have described a method for the isolation of a starch grain fraction from potato tubers. The tissue was first homogenized for 30 sec in a Waring Blendor with a medium containing 0.5 M sucrose. After filtration, the suspension was centrifuged at 750g. The pellet, after two washings, was termed the "starch grain fraction." Major contaminants were cell wall fragments, nuclei, and mitochondrial aggregates.

A more recent method for starch grain isolation was described by Rees and Duncan (2). After maceration by means of a "workshop-constructed steel abrasive-encased wheel rotating at high speed" the solution (in 18-20% glycerol) was poured through a 60-mesh and then a 120-mesh BSS sieve. It was claimed that these retained most of the cell debris. After centrifugation at 500g for 1 min and one washing the pellet was reported to contain starch grains with minimal contamination from other organelles or cytoplasm. However, no evidence was presented to substantiate this. The presence or absence of marker enzymes such as succinate dehydrogenase (for mitochondria) was not reported.

A new method is, therefore, necessary in order to obtain pure preparations of amyloplasts fractionated additionally, as we wanted, on grounds of size.

Most methods for purification of organelles utilize differential centrifugation followed by separation on a density gradient. For this technique to be successful a medium of density greater than that of the organelle to be purified must be available. Concentrated solutions of sucrose, sorbitol, or glycerol are generally found to be most suitable. Amyloplasts,

however, differ both in size and density, and while 80% sucrose will support the smallest and least dense of these under a low centrifugal field it will not do so with those in the highest density and/or size range.

Dilute solutions of colloidal silica (Ludox TM) have been used successfully in the isolation of nuclei (3) and chloroplasts (4). Its advantages include those of low viscosity, a negligible osmotic factor, and a high density ( $1.38 \text{ g/cm}^3$  at  $25^\circ\text{C}$ ). It is stable at  $4^\circ\text{C}$ .

The triiodated radiological contrast agent Urografin also has a high density and low viscosity. Its very low toxicity suggests also that it might have a minimal effect on the enzyme activity of the tissue to be fractionated. Urografin density gradients have been used successfully for the isolation and characterization of functional mitochondria (5) and for the separation of sporulating forms of bacteria (6).

A further advantage of Urografin and Ludox in the isolation of amyloplasts is that they do not contain carbohydrate. The usual alternatives are sucrose and sorbitol, and since the major biological function of amyloplasts is in carbohydrate synthesis and metabolism it is often desirable to exclude carbohydrate from the medium.

The properties of these two solutions, therefore, suggested that they might be suitable for the separation of amyloplasts by the technique of density gradient fractionation.

The method below describes the fractionation of amyloplasts from mature potato tubers.

## MATERIALS AND METHODS

Fresh potatoes (*Solanum tuberosum* L.) were bought locally. For much of the work Pentland Dell was used. Other varieties included Kerr's Pink and Record.

Ludox TM (colloidal suspension of silica) was supplied by E. J. du Pont de Nemours and Co., Inc., Industrials and Biochemicals Department, Wilmington, DE.

Urografin 76% (2 g sodium and 13.2 g methyl glucamine salts of 3, 5-diacetyl-amino-2, 4, 6-triiodo-benzoic acid in sterile 20-ml ampoules) was supplied by Schering A.G. Berlin.

Seventy-five grams of peeled and sliced tissue was soaked for 2 min in 0.1% sodium dithionite, and then thoroughly washed with distilled water. All subsequent operations were carried out at  $4^\circ\text{C}$ . The slices were then homogenized with 30 ml water in a Waring Blendor (15 sec at half speed, 5 sec at full speed). The resulting suspension was filtered through four layers of muslin and the filtrate centrifuged at 500g for 1 min. The supernatant solution was quickly decanted and the inside of the centrifuge tube cleaned with absorbent tissue. This removed cell



debris adhering to the sides of the tube. The pellet remaining consisted of a mixture of large and small amyloplasts.

After one washing with distilled water the mixed amyloplast pellet was resuspended in 4 ml water and 0.6 ml layered on top of a discontinuous Ludox or Urografin gradient.

Both gradients were prepared in transparent 5-ml cellulose nitrate centrifuge tubes. The Ludox TM gradient consisted of successive 1.3 ml volumes of 90%, 88%, and 84% Ludox TM in water. The Urografin gradient was successive 1.3-ml volumes of 70%, 65%, and 60% Urografin in water. After careful application of the sample both gradients were spun at 2500*g* for 10 min. With Ludox TM it was occasionally necessary to spin as high as 4000*g* to obtain the required separation.

Fractions were removed from the gradients using Pasteur pipets, centrifuged, and any pellet examined under the light microscope after staining for starch with I<sub>2</sub>/KI solution. Photographs were taken at a constant magnification of 250 diam. Amyloplast dimensions were measured using a stage graticule calibrated in 2- $\mu$ m subdivisions.

Succinate dehydrogenase was assayed using an oxygen electrode (7). DNA, RNA, and protein were determined in the washed amyloplast pellet as described by Rozijn and Tonino (8). A control of purified potato starch was run simultaneously. Nucleoside diphosphates were assayed colorimetrically using the coupled reaction with phosphoenolpyruvic acid and pyruvate kinase (9). Crystalline pyruvate kinase (type II), from rabbit skeletal muscle, was supplied by the Sigma Chemical Company, St. Louis, MO 63178. Starch synthetase (UDP dependent) was measured by the method of Leloir (10) and the UDP released determined as described above. In the controls UDP-glucose was added at the end of the 2-hr incubation. Dry weights were determined at each stage in the fractionation by drying in an oven at 60°C to constant weight.

## RESULTS AND DISCUSSION

Figure 1(a) shows the mixed population of washed amyloplasts before separation on the density gradient. Two types of amyloplast, large and small, are visible. The large ones are elliptical with a long axis of about 80  $\mu$ m and about 50  $\mu$ m in width. The small ones are more variable in size but seem to be generally circular with a diameter of around 20  $\mu$ m. Visible contamination was low and restricted to long fragments of cell wall material. No intact nuclei were seen. Since it is difficult to distinguish between a fully mature amyloplast and a membrane-free starch grain even in the electron microscope the relative proportions of amylo-

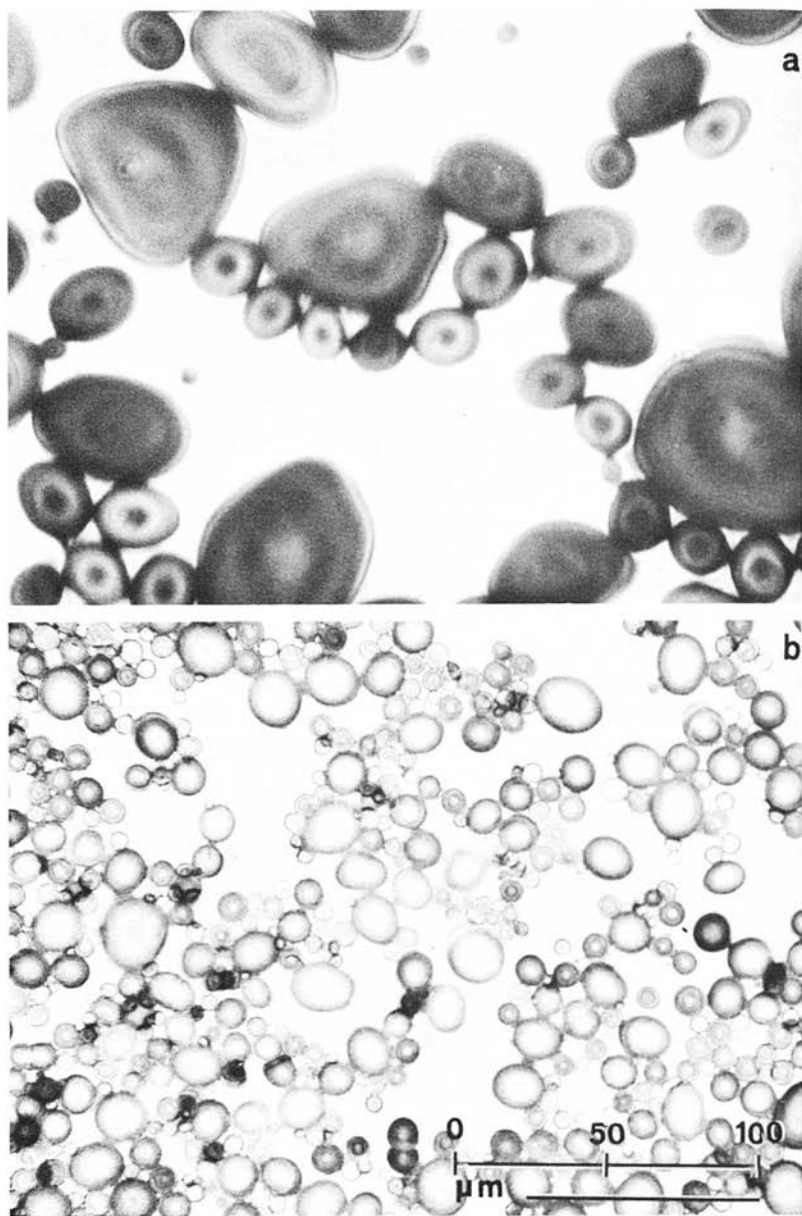
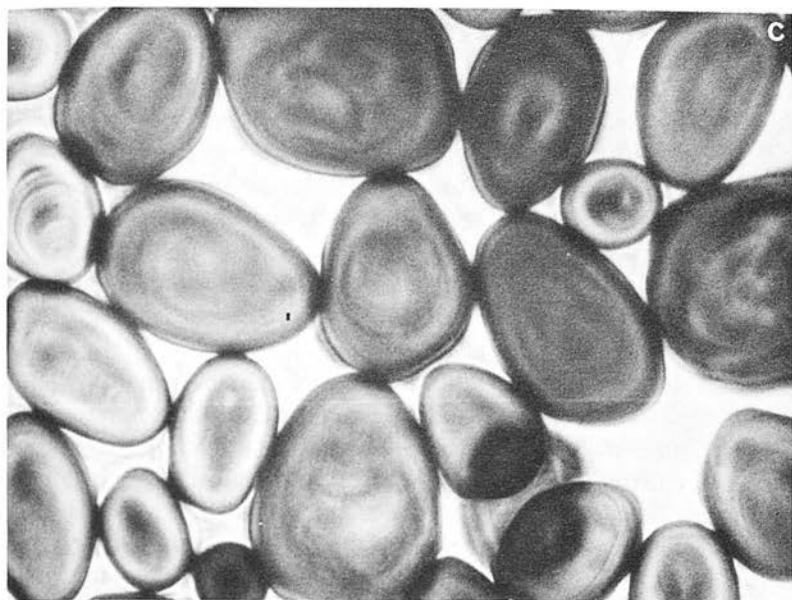


FIG. 1. (a). Once-washed potato amyloplasts before gradient fractionation. (b). Small amyloplast fraction from Ludox TM gradient. (c). Large amyloplast fraction from Urografin gradient.



plasts and starch grains in the once-washed pellet are not shown. The pellet was thus arbitrarily termed "amyloplasts."

The pellet was additionally examined for possible mitochondrial and nuclear contamination. Succinate dehydrogenase, a marker for mitochondrial activity was not present in the resuspended pellet.

Some DNA was associated with the once washed pellet. The amount varied but was generally in the range 15–30  $\mu\text{g}$  DNA/pellet. Part, if not all, of this DNA may be due to nuclear fragments associated with the amyloplast fraction. Dry weights of the various fractions are shown in Table 1. While it might have been more satisfactory to use total carbohydrate rather than dry weight as a measure of the amount of amyloplasts present, the method described by Morris (11) using the anthrone

TABLE 1  
DRY WEIGHTS<sup>a</sup> OF TISSUE FRACTIONS

Tissue fraction	Dry weight
Starting material (75 g fresh weight)	16.5 g (3)
Mixed amyloplast pellet	0.80 g (3)
Mixed amyloplast sample applied to each gradient	190 mg (3)
Small amyloplast pellet (from Ludox gradient)	31 mg (4)
Large amyloplast pellet (from Urografin gradient)	68 mg (4)

<sup>a</sup> The number of determinations is shown in brackets.

reagent showed considerable variability. This was at least partly due to the large dilution factors required.

Considerable amounts of RNA were also associated with the once-washed amyloplast pellet. Again this varied but was in the range 150–300  $\mu\text{g}$  RNA. A large proportion of this RNA can probably be attributed to the presence of cytoplasmic material.

Also present in the pellet were fairly high levels of nucleoside diphosphates (1.5–2  $\mu\text{m}$  moles per pellet).

After centrifugation of the mixed population on the Ludox gradient amyloplasts were distributed such that the smallest remained at the top and the largest accumulated at the 88/90% interface.

The small amyloplast fraction was present in a sharp and well-defined layer at the junction of 84% Ludox TM and the original amyloplast suspension. Figure 1(b) shows a representative sample of these after removal and centrifugation. It can be seen that a range of sizes is present, the maximum, however, being much less than that of the largest Fig. 1(a) and (c). Inspection of the top layer revealed a large number of extremely small (up to 3- $\mu\text{m}$  diameter) iodine-staining bodies. These may be proplastids or fragments of large amyloplasts.

The pellet of small amyloplasts was free from visible contamination. However, a small amount of RNA (15–20  $\mu\text{g}$ /pellet) still remained. No DNA was found to be present. The nucleoside diphosphate level was still significant and was in the range 0.05–0.10  $\mu\text{moles}$ /pellet.

While a good separation of small amyloplasts could be obtained with Ludox TM, it did not prove possible to obtain a concomitant purification of large ones. The layer of large amyloplasts at the 88/90% interface, while present in good yield was associated with a number of small amyloplasts.

Homogeneous preparations of the large amyloplasts in good yield were obtained from the Urografin density gradient. These banded at the 65/70% Urografin interface. Figure 1(c) shows a representative sample of these after removal from the gradient and centrifugation. The organelles here are almost uniform in size and shape and appear to be uncontaminated with cell debris. The nucleoside diphosphate level, however, was still significant and in the same range as that reported for the small amyloplasts. Thus, while some of the nucleoside diphosphates are washed off during fractionation on the gradient, it appears that at least some may be more strongly bound. This is in agreement with the results of Rees and Duncan (2).

It is interesting to note that the order of separation is reversed on the Urografin gradient. While some very small plastids remain in the top layer, the pellet at the bottom of the tube contains both large and small amyloplasts. Ludox behaves as a classical density gradient but Urografin seems to have additional properties.

Urografen and Ludox have been used mainly for the isolation and purification of cells and organelles. Whether Urografen interferes significantly with subsequent methods of analysis and/or the biological function of the tissue has not been extensively investigated. In describing the use of Ludox in purification of nuclei (3) and chloroplasts (4) the possibility of such interference was not considered to be a serious problem. Certainly neither Ludox (12) nor Urografen (6) is toxic to whole cells but presumably this reflects only their inability to penetrate the cell rather than a negligible effect on intracellular metabolism. It has been reported, however, that mitochondria isolated on a Ludox TM gradient exhibit respiratory control and are capable of high rates of respiration (13). On the other hand, Schmitt *et al.* (14) reported altered or no activities of selected enzymes in silica-gradient-purified unbroken chloroplasts. Silica sol gradients were, however, recommended for the isolation of pure high-molecular-weight substances from cell organelles.

The starch synthetase activity of the once washed amyloplasts (5–9  $\mu$ moles UDP released/ 2hr/ pellet) was not inhibited by preincubation in either Ludox or Urografen at the concentrations used on the gradient. Samples containing Ludox can give rather a high blank value when the enzyme is assayed using pyruvate kinase (8). Little difficulty should be encountered when using a radioactive method (9). In fact, much of the starch synthetase activity of the once-washed amyloplasts remained on the top layer of the gradient after centrifugation.

Urografen interferes with both the Folin (15) and biuret (16) methods for protein estimation. Thus, Urografen must either be removed from the pellets by washing, before protein can be assayed, or different methods of protein measurement must be considered. For example the direct spectrophotometric method of Warburg (17) may be suitable. Ludox does not interfere with either the Folin or biuret methods. Urografen also interferes with RNA estimation as it absorbs strongly at 260 nm.

In conclusion, both these materials give excellent separation of potato amyloplasts. Urografen may be less valuable than Ludox because of the difficulties in estimation of protein and RNA in its presence. It has proved possible, however, to isolate fairly pure preparations of large amyloplasts on a Ludox gradient. The separation was never as satisfactory as that with Urografen.

### ACKNOWLEDGMENT

We thank Dr. J. H. Duffus for suggesting the possible application of Urografen and Ludox to the separation of amyloplasts.

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## Changes in major element composition of developing barley grain

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### SUMMARY

Changes in the amounts of nitrogen, phosphorus, magnesium, calcium and sodium present in intact grain, endosperm, testa pericarp and embryo throughout the developmental period were recorded. While the overall pattern in intact grain was one of steady accumulation, considerable variation from this pattern was found within the different parts. The results are discussed in relation to the biochemical events accompanying maturation.

### INTRODUCTION

Much is now known of the biochemical changes taking place during development in cereal grains. The gross changes in the major storage materials such as protein and carbohydrate have been recorded by Jennings & Morton (1963*a*). The ultrastructure of the developing barley and wheat endosperm has been described by Buttrose (1960, 1963). The biochemistry of starch synthesis has been described in a series of papers by Baxter & Duffus (1971, 1973*a, b*). Similar studies have been undertaken for rice and maize (Baun *et al.* 1970; Ozburn *et al.* 1973).

Little recent information is available, however, on the associated changes in amounts of mineral elements during barley grain development. Brechley (1912) has followed the changes in nitrogen, ash and phosphoric acid during maturation in barley. The results were obtained for intact barley grains together with their associated glumes and paleae but with the awns removed. No attempts were made to separate the grains or the component parts. Changes in nitrogen content of whole wheat grains as well as of the separated endosperm, testa pericarp and embryos during development have been described by Jennings & Morton (1963*a*). Phosphorus changes in the separated grain parts have also been recorded (Jennings & Morton, 1963*b*). Little is known of the variation in amount during normal grain development of the mineral elements magnesium, calcium, potassium and sodium.

The role of mineral elements in plant metabolism has been reviewed by Hewitt & Smith (1975) and it is clear that many of the processes concerned in the deposition of macro molecules in the developing

grain require the presence of these elements. Other vital processes such as those concerned in nutrient transport require the participation of mineral elements.

The purpose of the present study, therefore, was to follow the changes in amount of major elements with time after fertilization in immature barley grain together with its component parts and to correlate these changes, where possible, with the known biochemical events accompanying growth and deposition of nutrient storage material.

### MATERIALS AND METHODS

#### *Plant material*

The two-row barley *Hordeum distichum* (L) Lam. cv Julia was used. The plants were grown under field conditions on the School of Agriculture farm at Bush, Midlothian (Low Fulford). The fertilizer application rate was 251 kg/ha of a compound containing N, P<sub>2</sub>O<sub>5</sub> and K<sub>2</sub>O in the ratio 23:11½:11½. Soil analysis after the fertilizer application showed that the amounts of available N, P and K in the soils were adequate for normal crop growth; soil pH was 6.4.

The method used to determine the date of anthesis was that of Merritt & Walker (1969). Grain was stored at -18 °C until analysis was carried out. Measurements were completed within 2 months of harvesting.

#### *Preparation of samples for analysis*

Grains were removed individually from the ear and the glumes and paleae removed, leaving the testa pericarp as the outer layer. These grains are referred to subsequently as 'intact' grains. Preparations of pericarp, embryo and endosperm were



made by removing each tissue separately by hand dissection. The testa pericarps, which were easily obtainable from 7 days after anthesis, were carefully removed in order to avoid contamination with aleurone. Preparations of testa pericarp free from endosperm were thus obtainable although in the later stages care was required to keep contamination by adhering glumes and paleae to a minimum. It was not possible to obtain sufficient embryos for analysis before 18–20 days after anthesis. Embryos were easily removed intact from the grain, after removal of the testa pericarp, as the scutellum is only weakly attached to the maternal tissue. Thus the embryo fraction also contained the scutellum. There was no contamination of embryos with endosperm as the tissues bounding the endosperm remained intact after scutellum removal. The aleurone layer was not separated from the endosperm, thus the results for endosperm include this tissue.

#### Major element analysis

Plant material was previously dried overnight at 80 °C. Four ml of a 0.35% (w/v) solution of selenium in conc.  $H_2SO_4$  was then added and the mixture heated gently for 10–15 min and allowed to stand, usually overnight. One ml of 30% hydrogen peroxide solution (AR grade) was then added, the mixture allowed to cool and another 1 ml of 30% hydrogen peroxide added. This was then digested vigorously for 1 h after the solution had cleared. The mixture was then made up to a known volume with glass-distilled water. For intact grains, 1 ml of solution corresponded to 10 grains, 3 and 5 days after anthesis. As the grains enlarged and contained more material, the volume was increased so that for the samples taken 50 days after anthesis 1 ml of solution corresponded to 0.16 grains. Similarly for testa pericarps, embryos and endosperms at 3 and 5 days after anthesis 1 ml of solution corresponded to 20 grains and from 50 days after anthesis 1 ml of solution corresponded to 0.32 grains. The appropriate blanks for each concentration were subtracted from the final results.

Phosphorus was determined colorimetrically by formation of the yellow phosphovanadate complex (Varley, 1966). Sodium and potassium were determined by flame photometry using lithium as an internal standard. Calcium and magnesium were determined by atomic absorption spectrophotometry in the presence of 2500 mg lanthanum/kg to eliminate interference from phosphorus. Nitrogen as ammonium was determined colorimetrically using the salicylate-dichloroisocyanurate reaction in the presence of nitroprusside (Crooke & Simpson, 1971).

The results are expressed in micrograms of the

element per grain or per embryo, testa pericarp or endosperm.

#### Dry-weight determination

Dry weights were determined for intact grains by drying a suitable number to constant weight at 80 °C.

### RESULTS

#### Variation in fresh and dry weight of intact grain during maturation

The curves in Fig. 1 describe the growth of individual intact grains in terms of fresh and dry weight. Growth, initially slow, accelerated more rapidly between 20 and 35 days after anthesis. Dehydration commenced around 45 days and continued until maturity when the moisture content was around 18%.

#### Variation in the amount of nitrogen and phosphorus in intact grains, endosperms and testa pericarp throughout development

The results, expressed in  $\mu g/\text{grain}$  or per thereof, are shown in Fig. 2. Nitrogen accumulated rapidly in the intact grains and endosperms over the development period. The pattern of accumulation was similar in both and showed a slight decrease after 45–50 days. About 75–80% of total grain nitrogen was associated with the mature endosperm, the remainder being accounted for by the testa pericarp and embryo. Nitrogen in the testa pericarp varied little between 15 and 30 days. Thereafter it fell steadily to reach a very low value at maturity. Phosphorus accumulated slowly but steadily in both endosperm and intact grain over the maturation period. In the testa pericarp, as with nitrogen, the levels of phosphorus fell steadily

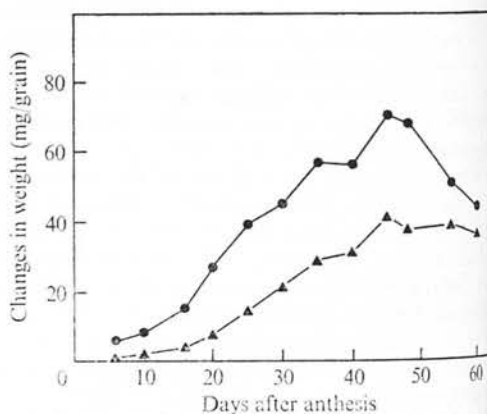


Fig. 1. Variation in fresh and dry weight of intact grains during maturation. ●—●, Fresh weight; ▲—▲, dry weight.

Fig. 2. V phosphorus; testa pericarp; Nitrogen; from 30 d The resul anthesis t was locat Variation magnesiu. The re present rapidly o accumula anthesis. always g Calcium: out matu lated slov

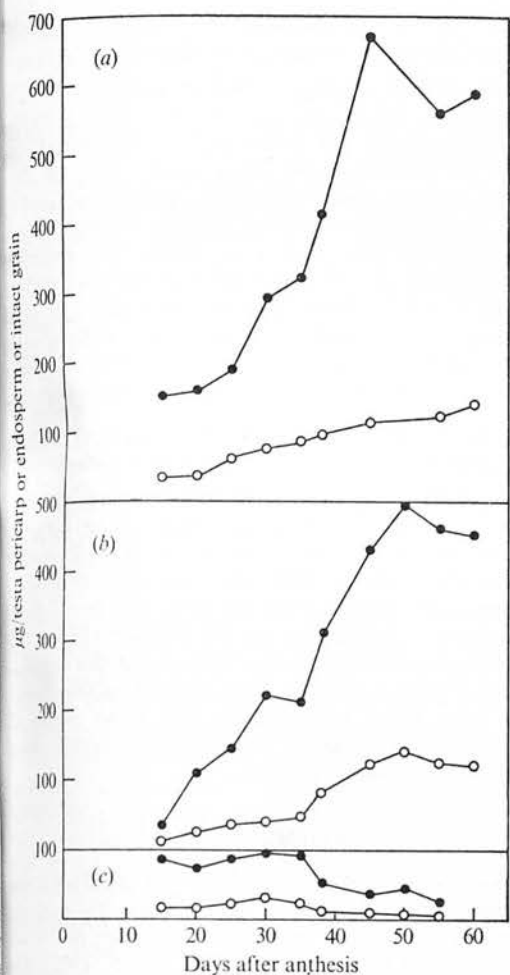


Fig. 2. Variation in the amounts of nitrogen and phosphorus in intact grains (a), endosperms (b), and testa pericarps (c), throughout development. ●—●, Nitrogen; ○—○, phosphorus.

from 30 days after anthesis to very low final levels. The results indicate that from about 38 days after anthesis the major part of intact grain phosphorus was located in the endosperm.

#### Variation in the amounts of potassium, calcium, magnesium and sodium in maturing intact grains

The results are shown in Fig. 3. Potassium is present in greatest amount, and accumulates rapidly over the developmental period. The rate of accumulation decreased slightly from 45 days after anthesis. The amount of potassium present was always greater than the amount of phosphorus.

Calcium and magnesium were present throughout maturation in similar amounts and accumulated slowly but steadily. The amount of sodium

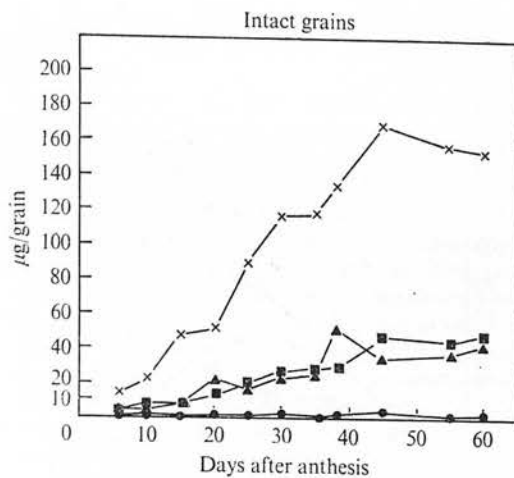


Fig. 3. Variation in the amounts of potassium, calcium, magnesium and sodium in intact grains throughout development. x—x, Potassium; ■—■, magnesium; ▲—▲, calcium; ●—●, sodium.

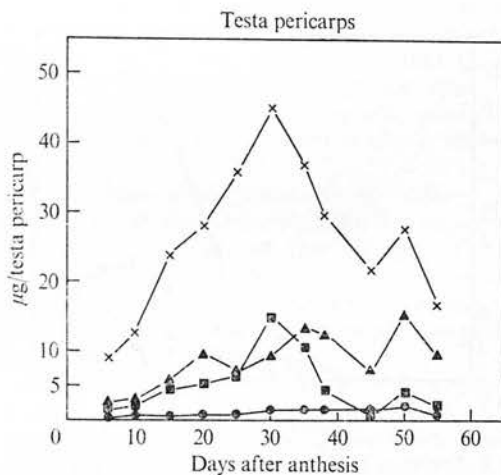


Fig. 4. Variation in the amounts of potassium, calcium, magnesium and sodium in the testa pericarp throughout development. x—x, Potassium; ■—■, magnesium; ▲—▲, calcium; ●—●, sodium.

changed little over the first 35 days, remaining at about 1–2 µg/grain by 60 days.

#### Variation in the amounts of potassium, calcium, magnesium and sodium in the testa pericarp over the maturation period

The results are shown in Fig. 4. Again, potassium was present in greatest concentration and accumulation was initially rapid. A peak was reached at 30 days after anthesis and the tissue thereafter

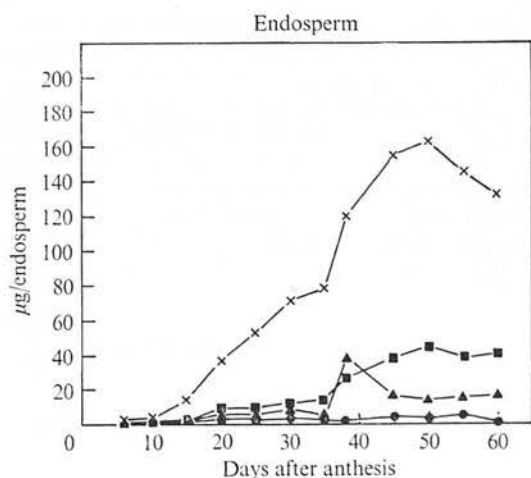


Fig. 5. Variation in the amounts of potassium, calcium, magnesium and sodium in endosperm throughout development. x—x, Potassium; ■—■, magnesium; ▲—▲, calcium; ●—●, sodium.

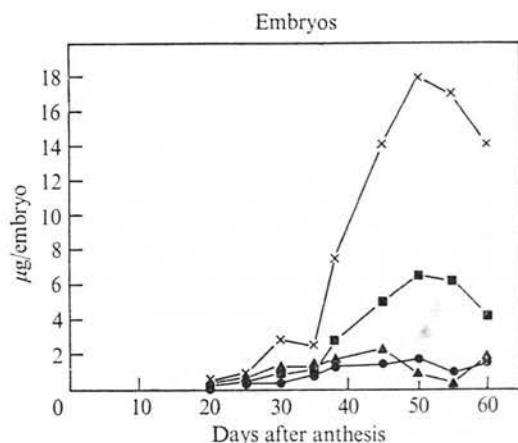


Fig. 6. Variation in the amounts of potassium, calcium, magnesium and sodium in embryos throughout development. x—x, Potassium; ■—■, magnesium; ▲—▲, calcium; ●—●, sodium.

lost potassium at a considerable rate, falling to  $17 \mu\text{g}/\text{testa pericarp}$  at maturity. While the pattern of accumulation of calcium and magnesium was initially similar, their paths diverged after 25–30 days. For calcium the overall pattern was one of accumulation, whereas for magnesium a peak was reached at 30 days with subsequent rapid losses to reach a level of  $2 \mu\text{g}/\text{testa pericarp}$  at maturity. Sodium increased slowly in amount over the maturation period reaching a maximum level by 50 days after anthesis. Amounts present were low but relatively higher than in the intact grains.

#### Variation in the amounts of potassium, calcium, magnesium and sodium in developing endosperm

The results are shown in Fig. 5. The pattern of potassium accumulation was very similar to the overall pattern for intact grain. The initial value was low but increased more rapidly from 35 days after anthesis and a maximum value was reached by 50 days. Calcium and magnesium levels were initially low but present in significant amounts. Magnesium levels increased steadily to reach a plateau by 45 days. Calcium remained low until 35 days when it rose to a small peak at 38 days and then fell off to constant levels of  $14 \mu\text{g}/\text{endosperm}$  thereafter. Sodium levels remained low throughout.

#### Variation in the amounts of potassium, calcium, magnesium and sodium in developing embryos

The results are shown in Fig. 6. After an initial lag phase from 20 to 35 days after anthesis, potassium levels rose markedly until 50 days after anthesis. Levels fell off slightly thereafter. Magnesium levels increased rapidly after 35 days, reaching a maximum at 50 days. Calcium and sodium levels were similar throughout the developmental period showing their highest values around 45 days after anthesis. In the later stages of embryo development magnesium levels were 2–3 times those of calcium.

### DISCUSSION

The change in water content of the intact grain throughout maturation was similar to that described for barley endosperm (Baxter & Duffus, 1973a) and embryo (Duffus & Rosie, 1975). The pattern of results was also similar to that described for wheat, by Jennings & Morton (1963a). Corresponding results for barley testa pericarp are not described here. Observation suggests, however, that this tissue has maximum water content around 20 days after anthesis. At this stage the tissue has maximum metabolic activity which thereafter decreases rapidly as it shrinks and dries (Duffus & Rosie, 1973a).

At the beginning of maturation the grain consists for the greater part of pericarp tissue. As the grain develops, the endosperm grows rapidly as it accumulates starch, and the pericarp decreases progressively in size. Thus at 15 days after anthesis the pericarp contained 56% of intact grain nitrogen and 50% of phosphorus. By 40 days, however, these figures had fallen to 10% of nitrogen and 10% of phosphorus. The endosperm, on the other hand, at 15 days contained only 23% of intact grain nitrogen and 30% of phosphorus. By 40 days, however, this tissue contained around 90% of intact grain phosphorus and 70% of nitrogen.

The fate of the pericarp tissue during the stages of a mechanical test pericarp is not observed. The proportion is similar to the enzyme the earlier may reflect the later accumulation of endosperm deposits. The change in agreement Morton (were not). As with total potassium pericarp about 40 rapid decrease is as in embryo, the embryo, and so it transfer embryo n postulate. The n requires e testa pericarp considera Potassi starch syn However, tion is n anthesis v is low. It starch syn the rapid

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The fate of the nitrogen and phosphorus lost from the pericarp tissue during the middle and later stages of maturation is unknown. It is possible that a mechanism may exist for its transfer from the testa pericarp to the endosperm. Such losses were not observed by Jennings & Morton (1963*a*) in similar experiments with wheat.

The increase in nitrogen, of which a major portion is presumably protein, is associated with a similar rapid increase in the activity of many of the enzymes present in the developing grain. In the earlier stages, up to 30 days after anthesis, this may reflect a synthesis of enzyme protein while in the later stages it may be associated with the accumulation of protein bodies in the starchy endosperm (Buttrose, 1960) and perhaps also with the deposition of latent forms of such enzymes as  $\beta$ -amylase (Duffus & Rosie, 1973*b*).

The changes in phosphorus content are in broad agreement with those described by Jennings & Morton (1963*b*) for wheat although pericarp losses were not observed.

As with nitrogen and phosphorus much of the total potassium of the grain is present initially in the pericarp. However, by 30 days after anthesis about 40% of potassium remains in this tissue. A rapid decrease in total pericarp potassium at 30 days is associated with a similar but rapid increase in embryo and endosperm potassium. The gains to the embryo are similar to losses from the pericarp and so it may be that a mechanism whereby direct transfer of nutrients from the pericarp to the embryo may exist. A similar mechanism might be postulated for magnesium.

The maintenance of cell potassium levels requires energy thus it is not surprising that as the testa pericarp ages and metabolic activity declines considerable losses of potassium are observed.

Potassium may have a role as a cofactor in the starch synthetase reaction (Nitsos & Evans, 1969). However, in endosperm the rate of starch deposition is maximal between 10 and 15 days after anthesis while the rate of potassium accumulation is low. It may be that potassium supply is limiting starch synthesis in endosperm. On the other hand, the rapid increase in embryo potassium at 35 days

can be closely correlated with similar rapid increase in embryo total carbohydrate (Duffus & Rosie, 1975).

While the overall pattern of accumulation in intact grain of both magnesium and calcium is similar there is considerable variation between the individual tissues examined. Testa pericarp magnesium falls almost to zero after a maximum at 30 days, while endosperm magnesium accumulates steadily over the maturation period. Magnesium is a cofactor for a number of enzyme-catalysed reactions and its loss from the testa pericarp may well be correlated with the decline in metabolic activity of that tissue. The gains in magnesium of both endosperm and embryo during the later stages may have been at the expense of the testa pericarp.

A feature of embryogenesis in rye (*Secale cereale* L.), and therefore possibly also in barley, is the appearance of large numbers of ribosomes (Hallam, 1972). The increase in embryo magnesium levels may well be associated with functional ribosome assembly.

Calcium, on the other hand, accumulates steadily and slowly. In most plants calcium is relatively immobile and in the cereal grain exists at least partly in combination with phytic acid. The absence of phytase activity would presumably prevent any transport of calcium within the grain itself.

While measurable amounts of sodium were present in all tissues throughout the maturation period, the function of this element in plant metabolism is little understood. It is interesting to note that the embryo contains relatively the greatest amount of sodium, the levels occasionally exceeding those of calcium.

Thus, while the overall pattern of accumulation of the major elements in developing barley grain has been established, it is clear that much remains to be elucidated about their biochemical function and their transport into and within the grain.

The authors wish to thank Dr P. Crooks and Mr A. Scott for carrying out the analyses.

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## Changes in trace element composition of developing barley grain

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(Received 27 October 1975)

### SUMMARY

Changes in the amounts of the trace elements iron, zinc, manganese and copper, present in intact grain, endosperm, testa pericarp and embryo throughout the developmental period were recorded. Accumulation of iron and zinc in intact grain was extremely rapid, rising in the case of iron from 40 ng/grain to 1600 ng/grain between 5 and 40 days after anthesis. Manganese accumulated steadily throughout, reaching a final value of 475 ng/grain. Significant amounts of copper were present throughout and a small peak was observed at 38 days after anthesis. Some variation from this overall pattern was found in the different morphological parts as development proceeded. This was notable in the case of embryo manganese, which was undetectable until 45 days after anthesis, when levels rose dramatically to reach a steady maximum of around 100 ng/embryo. The results are discussed in relation to the biochemical events accompanying maturation.

### INTRODUCTION

Previous work has described the variation in amounts of the major elements present during the development of barley grain and its component parts (Duffus & Rosie, 1976). While the overall pattern in intact grain was one of steady accumulation, considerable variation from this pattern was found within the different grain parts such as the endosperm, testa pericarp and embryo. The results were correlated with the known biochemical events accompanying growth and deposition of grain storage material. Little is yet known, on the other hand, of the variation in amounts of trace elements during seed development. Certainly a number of enzymes which require trace elements for activity such as inorganic pyrophosphatase (manganese) and peroxidase (iron) are present in immature barley grain (Baxter & Duffus, 1973; Duffus, 1970). Grain development must therefore be regulated by the supply of trace elements to the ear.

The purpose of the present study was to describe the variation in amounts of the selected trace elements, iron, zinc, manganese and copper, in developing barley grain and its component parts and to correlate the results, where possible, with the known biochemical events accompanying grain growth and maturation.

### MATERIALS AND METHODS

#### *Plant material*

The two-row barley *Hordeum distichum* (L.) Lam. cv. Julia was used. The plants were grown under field conditions on the School of Agriculture farm at Bush, Midlothian (Low Fulford). The fertilizer application rate was 251 kg/ha of a compound containing N, P<sub>2</sub>O<sub>5</sub> and K<sub>2</sub>O in the ratio 23:11½:11½. Soil analysis after the fertilizer application showed that the amounts of available N, P and K in the soils were adequate for normal crop growth; soil pH was 6.4.

The method used to determine the date of anthesis was that of Merritt & Walker (1969). Grain was stored at -18 °C until analysis was carried out. Measurements were completed within 2 months of harvesting.

#### *Preparation of samples for analysis*

Grains were removed individually from the ear and the glumes and paleae removed, leaving the testa pericarp as the outer layer. These grains are referred to subsequently as 'intact' grains. Preparations of pericarp, embryo and endosperm were made by removing each tissue separately by hand dissection. The testa pericarps, which were easily obtainable from 7 days after anthesis, were

carefully removed in order to avoid contamination with aleurone. Preparations of testa pericarp free from endosperm were thus obtainable although in the later stages care was required to keep contamination by adhering glumes and paleae to a minimum. It was not possible to obtain sufficient embryos for analysis before 18–20 days after anthesis. Embryos were easily removed intact from the grain, after removal of the testa pericarp, as the scutellum is only weakly attached to the maternal tissue. Thus the embryo fraction also contained the scutellum. There was no contamination of embryos with endosperm as the tissues bounding the endosperm remained intact after scutellum removal. The aleurone layer was not separated from the endosperm, thus the results for endosperm include this tissue.

#### Trace element analysis

Plant material, previously dried at 80 °C was ashed at 450 °C. After twice taking to dryness with 2 ml of 6 N-HCl, the material was digested for 30 min on a steam bath with 5 ml of 1.5 N-HCl. The filtered solution, in a known volume, was analysed for iron, zinc, manganese and copper by atomic absorption using a Unicam SP 90.

For intact grains, 1 ml of solution corresponded to 37.5 grains, 3 and 5 days after anthesis. As the grains enlarged, the volume was increased so that for the samples taken 50 days after anthesis 1 ml of solution corresponded to 0.3 grains. Similarly for testa pericarps, endosperms and embryos at 7 days after anthesis 1 ml of solution corresponded to 24 grains while at 55 days after anthesis 1 ml of solution corresponded to 1.3 grains. The appropriate blanks for each concentration were subtracted from the final results.

The results are expressed in ng of the element per grain or per embryo testa pericarp or endosperm.

### RESULTS

#### *Variation in the amounts of iron, zinc, manganese and copper in intact grains throughout development*

The results, expressed in ng/grain are shown in Fig. 1. Each trace element was detectable from as early as 5 days after anthesis. Iron, which is present in greatest amount, shows a steady increase to a maximum value at 40 days and thereafter falls off. Zinc accumulated rapidly reaching a maximum value of 1000 ng/grain at maturity. The rate of manganese accumulation was less than that of iron or zinc and reached a maximum value of 475 ng/grain by 50 days after anthesis. Copper was present in small but significant amounts throughout maturation, reaching a peak around 40 days after anthesis.

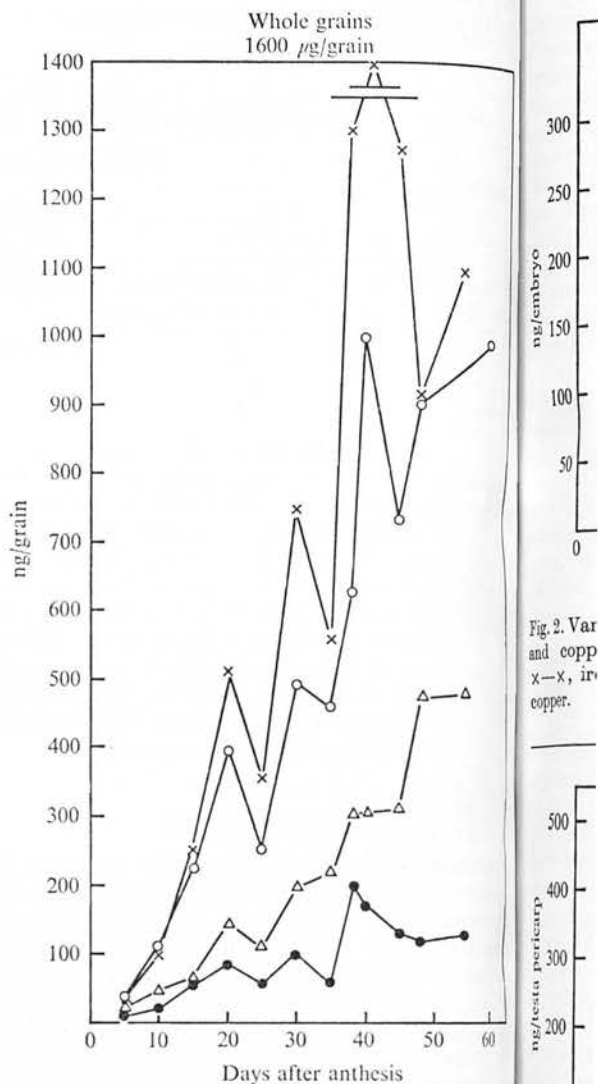


Fig. 1. Variation in the amounts of iron, zinc, manganese and copper in intact grains throughout development. x—x, iron; o—o, zinc;  $\triangle$ — $\triangle$ , manganese;  $\bullet$ — $\bullet$ , copper.

#### *Variation in the amounts of iron, zinc, manganese and copper in embryos throughout development*

The changes in the amounts of iron, zinc, manganese and copper throughout embryo development are shown in Fig. 2. The pattern observed for iron was similar to that in the intact grain with a maximum value of 340 ng/embryo at 45 days after anthesis. By maturity, however, this value had fallen to 130 ng/embryo. It is interesting to note that while significant amounts of zinc and copper were present at 20 days after anthesis, iron was not then detectable. The number of embryos used for analysis

Fig. 2. Variation in the amounts of iron, zinc, manganese and copper in embryos throughout development. x—x, iron; o—o, zinc;  $\triangle$ — $\triangle$ , manganese;  $\bullet$ — $\bullet$ , copper.



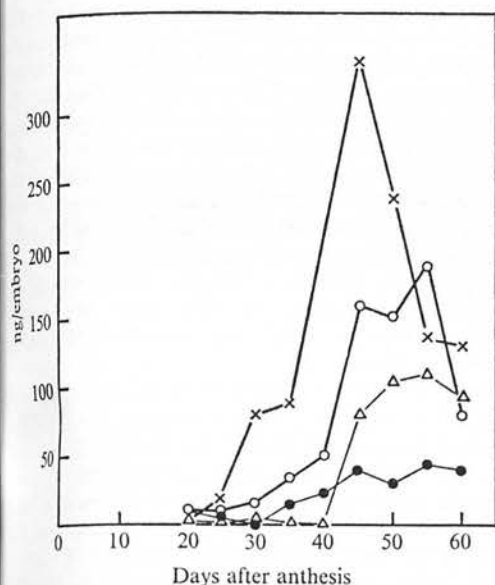


Fig. 2. Variation in the amounts of iron, zinc, manganese and copper in embryos throughout development. x-x, iron; o-o, zinc; Δ-Δ, manganese; ●-●, copper.

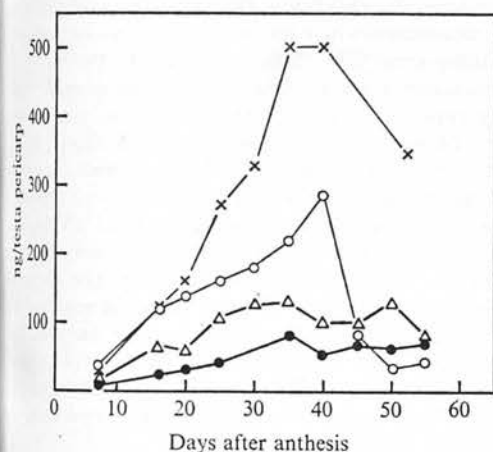


Fig. 3. Variation in the amounts of iron, zinc, manganese and copper in the testa pericarp throughout development. x-x, iron; o-o, zinc; Δ-Δ, manganese; ●-●, copper.

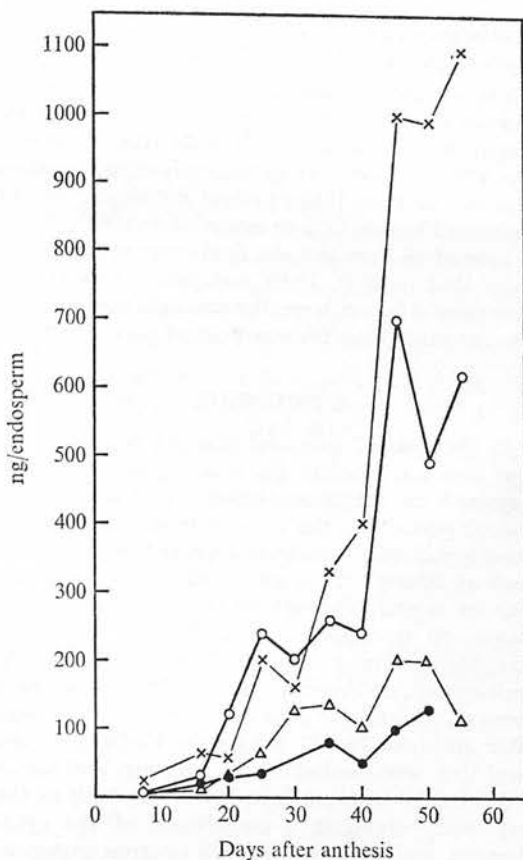


Fig. 4. Variation in the amounts of iron, zinc, manganese and copper in endosperm throughout development. x-x, iron; o-o, zinc; Δ-Δ, manganese; ●-●, copper.

give a final steady maximum value. Copper was present in small amounts and accumulated slowly throughout embryo development.

#### *Variation in the amounts of iron, zinc, manganese and copper in the testa pericarp throughout development*

The results are shown in Fig. 3. The amount of iron rose rapidly to reach a maximum value at 35-40 days after anthesis. The variation in concentration of zinc was noteworthy in that a sharp maximum was reached at 40 days, followed by a rapid decrease to very low levels. The ratio of iron to zinc in the period up to 40 days after anthesis was much less than for the developing embryo and similar to that for intact grain. After 40 days the ratio increased considerably. Manganese and copper showed similar patterns of low but steady accumulation over the maturation period.

*Variation in the amounts of iron, zinc, manganese and copper in endosperm throughout development*

The changes in amount of iron, zinc, manganese and copper throughout endosperm development are shown in Fig. 4. Iron and zinc rose slowly at first and then very rapidly to give maximum values at about 45 days. While present initially in similar amounts the rate of iron accumulation was faster at around 40 days and the final concentration was twice that of zinc. Both manganese and copper were present in much smaller amounts but accumulated steadily over the maturation period.

### DISCUSSION

In the case of zinc and manganese, translocation from the plant to the intact grain on the ear appeared to continue throughout the developmental period. In the case of iron, on the other hand, significant losses were apparent after the high peak at 40 days. To a lesser extent the same was true for copper. Throughout the early and middle stages of development translocated iron was distributed almost equally between the testa pericarp and endosperm with a significant amount going to the embryo as it developed from 20 days after anthesis. By 55 days only 15–20% of the total iron was located in the pericarp and about 7–8% in the embryo. Iron has a role both in the respiratory chain as a constituent of the cytochromes, and in photosynthetic electron transport as a constituent of the terminal electron acceptor ferredoxin. The testa pericarp changes from pale green to bright green as early as 10 days after anthesis and the tissue contains a large number of chloroplasts many of which contain starch (Duffus & Rosie, 1973). These disappear around 40 days after anthesis. An adequate supply of iron would therefore be required both for the generation of reducing power in the chloroplasts which is utilized in carbon dioxide fixation and starch synthesis, and for cytochrome synthesis or activity. Thus, as the pericarp matures and becomes metabolically inactive, iron is no longer required and supplies are directed almost exclusively to the endosperm. In this tissue apart from its role in supplying energy and reducing power for the biosynthesis of carbohydrate, protein and lipid, iron is required as a cofactor of the haemoproteins catalase and peroxidase which are known to be present in immature barley endosperm (Duffus, 1970). Peroxidase, which is present in high activity, may be concerned in the regulation of indole acetic acid, which in turn may play a part in regulating endosperm enzyme activity (MacLeod & Palmer, 1969). The notable decrease in the amount of iron in the intact grain after 45 days is difficult to explain. Losses are

almost accounted for by those in the embryo and testa pericarp. Previous work has shown similar losses of protein, DNA and RNA in the embryo after 50 days (Duffus & Rosie, 1975). This may be caused either by incomplete dissection and removal of embryo tissue at this stage or by hydrolysis and subsequent transport of nutrients out of the embryo. On the other hand the decrease in iron may be due to inhibition of its uptake by other ions. Both manganese (Somers & Shive, 1942) and zinc (Ambler, Brown & Gauch, 1970) which are accumulating rapidly at this time have been shown to interfere with translocation of iron in soya-bean plants.

At 7 days after anthesis 90% of grain zinc was associated with the testa pericarp. By 30 days this had fallen to 40% and by 55 days to around 5%. Absolute losses of zinc from the pericarp from 40 days coincided with striking increases in both endosperm and embryo zinc. This reinforces the suggestion, previously made (Duffus & Rosie, 1976), that a mechanism may exist whereby nutrients can be transferred from the pericarp to other parts of the grain. The accumulated zinc in these tissues may be utilized in the synthesis and activation of various enzymes such as carbonic anhydrase or alcohol dehydrogenase.

Manganese is required for a number of biological functions. In the testa pericarp, manganous ions are concerned in the evolution of oxygen by Photosystem II. The continued presence of manganese after photosynthesis has ceased probably explains why malic enzyme activity continues up to 55 days after anthesis (Duffus & Rosie, 1973).

The pattern of accumulation of manganese in the embryo is of particular interest. The increase in intact grain manganese over the final 15 days of development can be accounted for only by the gains in embryo manganese. Since the increase largely parallels that of zinc, which is required for carbonic anhydrase activity, it may be that this facilitates the synthesis of proplastid photosynthetic systems. Proplastids are visible at this time in root primordia of developing rye embryos (Hallam, 1972).

Manganese is also required for activity of a number of enzymes of which inorganic pyrophosphatase is one. This enzyme, which is concerned in the regulation of the equilibrium of uridine diphosphoglucose (adenosine diphosphoglucose) pyrophosphorylase is present in immature endosperm (Baxter & Duffus, 1973) and testa pericarp (Duffus & Rosie, 1973).

There is little variation between the tissues in the pattern of copper accumulation. Its presence is certainly required in the testa pericarp as a cofactor of plastocyanin, a constituent of the photosynthetic electron transport chain. It may also be

required for the activity of phenol oxidase an enzyme reported to be present in this tissue (A. R. Nutbeam, unpublished). As a constituent of cytochrome oxidase the presence of copper would be expected in all tissues capable of respiratory activity.

The different patterns of accumulation of selected trace elements have thus been established in developing grain during normal growth under field conditions. Their distribution within the grain

has also been described. Little is known, however, of the factors controlling their transport both into and within the grain and whether or not their rate of supply limited grain growth and development. As with the major elements much remains to be elucidated about their precise function in grain development.

The authors wish to thank Dr D. Purves and Mrs Janice Barr for carrying out the analyses.

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EVIDENCE FOR  $C_4$  PHOTOSYNTHESIS IN  
BARLEY PERICARP TISSUE

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**SUMMARY:** The products of photosynthetic carbon dioxide fixation were determined in isolated pericarps of immature barley grains. Of the carbon dioxide fixed after 1 min photosynthesis 84% was in the  $C_4$  acid malic acid. The remaining label was in hexose phosphates and sucrose. By 2 min sucrose was the major labelled product and at 6 min accounted for 94% of the total carbon dioxide fixed.

$C_3^*$  and  $C_4$  plants are associated with certain physiological and anatomical characteristics. For example, in comparison with  $C_3$  plants,  $C_4$  plants have a low carbon dioxide compensation point (1) and high rates of photosynthesis (2). In addition they have a specialised type of leaf anatomy (3) in which the central bundle sheath cells are surrounded by outer or mesophyll cells. The leaves of  $C_3$  plants have essentially a single type of cell specialising in photosynthesis. Thus, the temperate plants barley and wheat have been classified as  $C_3$ , and the tropical plants maize and sugar cane as  $C_4$ .

It is noteworthy, however, that this division of plants into groups is based entirely on physiological and anatomical properties of the leaves. It is well established, however, that the dry matter entering the grain of cereals is derived from photosynthesis after ear emergence (4) and that it is the ear itself, together with the flag leaf, which makes the major contribution to grain carbohydrate (5,6). Indeed at the time of ear emergence many of the lower leaves are senescent.

\*Abbreviations:  $C_3$  plants, plants which have only the reductive pentose phosphate pathway in photosynthesis;  $C_4$  plants, plants which have both  $C_4$  dicarboxylic acid pathway and reductive pentose phosphate pathway in photosynthesis; CAM plants, succulent plants with Crassulacean acid metabolism.

The tissues of the ear which contain chlorophyll and which may contribute directly to grain carbohydrate include the awns, glumes, paleae and pericarp. The awns are certainly capable of photosynthesis and of supplying carbohydrate to the grain (7) although under certain conditions awned plants yield less than non awned plants of the same variety (8). The glumes and paleae are dull green, loosely adhering protective tissues which surround the grain. They later become part of the husk of the mature grain. Nothing is known of their ability to fix and transport carbon dioxide into the immature grain. The pericarp of immature cereal grains is a bright emerald green tissue surrounding the outer cells or aleurone layer of the endosperm. It is contained in a transparent "bag" of tissue probably only one or two cells thick. Thus, the pericarp is distinct in structure and location from the leaves. If, additionally, it has different biochemical characteristics the situation could arise where the plant is  $C_3$  with respect to the leaves and  $C_4$  (for example) with respect to the pericarp. Previous work (9) has reported the presence, in the barley pericarp, of levels of phosphoenol pyruvate carboxylase (PEPC; EC 4.1.1.31) higher than those usually found in the leaves of  $C_3$  plants. This enzyme catalyses the primary carboxylation reaction in  $C_4$  plants. Phosphoenol pyruvate synthetase, an enzyme of the  $C_4$  dicarboxylic and pathway, was also reported to be present.

This work describes the metabolic events following carbon dioxide fixation by isolated pericarps. Results with leaves of maize ( $C_4$ ), pea ( $C_3$ ) and *Sedum spectabile* (CAM) are included for comparison.

#### MATERIALS AND METHODS

Plants were grown in greenhouses with natural daylength extended to 18 h with mercury vapour lamps. Barley grown in these conditions produced grain which reached maturity 60 days after anthesis. Ears were removed from the growing plants 25-30 days after anthesis, immediately prior to isolation of the pericarps. At this stage in development the grain was still increasing in size, both layers of the pericarp were fleshy and the chlorophyll content

of the green layer was at its maximum value (9). The paleae, lemma and transparent layer of the pericarp were peeled from the grain. The green layer was then carefully removed and quickly transferred to glass fibre discs and incubated with  $^{14}\text{CO}_2$  using a technique similar to that described by Coombs and Baldry (14). After incubation the pericarps were washed and then homogenised in ethanol at 70°C. The alcohol extract was centrifuged at 1,000 g for 2 min, and 5  $\mu\text{l}$  of the supernatant counted for radioactivity. The residue was re-extracted in water for 24 h and the total carbon dioxide fixed calculated from the sum of alcohol soluble and water soluble radioactivity. Chlorophyll was determined in the alcohol extract as described by Wintermans and De Mots (15), and the products of carbon dioxide fixation separated electrophoretically as described by Farineau (16).

#### RESULTS AND DISCUSSION

The rate of photosynthesis by pericarps is low compared to maize, pea and *Sedum* leaves (Fig.1). The low rates may in part be due to the difficulties encountered in preparing the tissue for such experiments. The chlorophyll containing layers of the pericarp are delicate and care must be taken to ensure a minimum of cell damage. Broken cells are then susceptible to further attack by the products of phenol oxidase activity. While the number of pericarps used was the minimum required to achieve sufficient incorporation to identify products, the time taken for their removal from the grain and transfer to glass fibre discs was much longer than that taken for leaf experiments. *In vivo* rates, therefore, are probably higher than those reported here.

In order to obtain measurable rates of photosynthesis with pericarps the partial pressure of carbon dioxide used was 0.1%. Under these conditions pea has a rate of photosynthesis similar to that of maize. Under physiological conditions (0.03% carbon dioxide) the rate of pea photosynthesis would be relatively much less.

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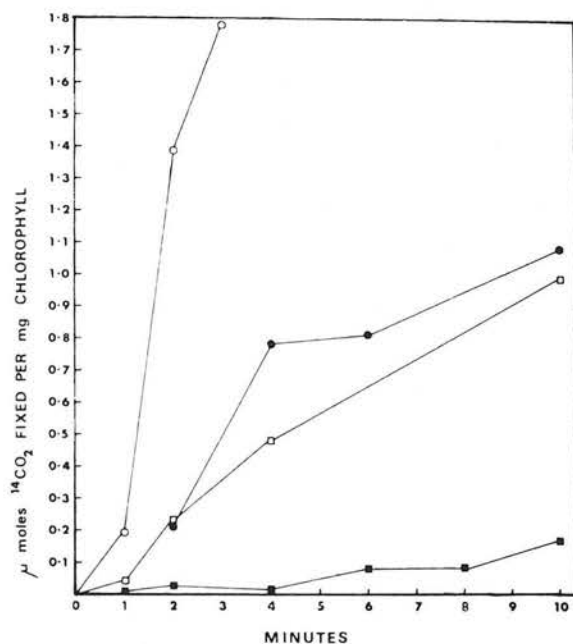


Fig. 1. Rate of carbon dioxide fixation by the leaves of *Sedum spectabile*, maize (*Zea mays* var. Golden Bantam), pea (*Pisum sativum* var. Meteor) and pericarps of barley (*Hordeum distichum* var. Julia).

Pericarps (10 per disc) were placed on glass fibre discs (21 mm diameter) soaked in isotonic buffer (50 mM tricine KOH buffer, pH 7.5; 0.33 M sorbitol) to prevent dehydration. Three such discs were placed in a Perspex chamber of 11 ml volume and 20  $\mu$ l of a 17 mM solution of sodium (<sup>14</sup>C) bicarbonate (specific activity approximately 60 Ci/Mole) were injected through a rubber seal into a well containing 0.15 ml lactic acid, such that the concentration of carbon dioxide in the chamber was 0.1%. The chamber was illuminated for periods of time from 1 minute to 10 minutes with a tungsten halogen lamp (incident light intensity 17,000 Lux) at 25°C.

○—○ *Sedum spectabile*; ●—● *Pisum sativum*; □—□ *Zea mays*;  
■—■ *Hordeum distichum* pericarp.

synthetic products (10). It was therefore surprising that, of the carbon dioxide fixed in barley pericarps after 1 min photosynthesis, 84% was in the C<sub>4</sub> acid malic acid (Fig. 2). The remaining label was in hexose phosphates and sucrose. By 2 min sucrose was the major labelled product and at 6 min accounted for 94% of the total <sup>14</sup>C fixed. Similar results have been obtained for wheat pericarp. At the same time normal C<sub>3</sub> photosynthesis was observed with pea (C<sub>3</sub>) leaves. These results for cereal pericarp closely resemble



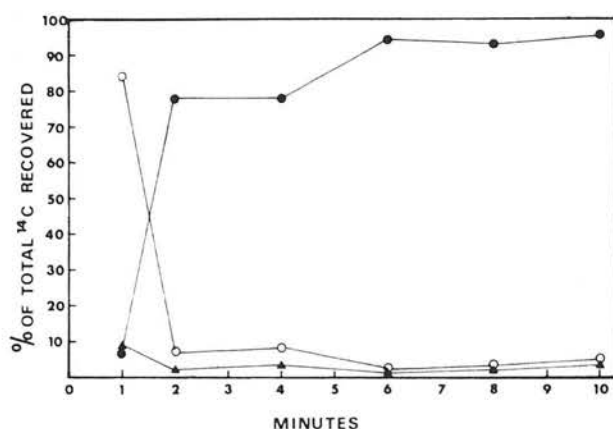


Fig.2. Changes in the distribution of radioactivity after exposure of pericarps to 0.1%  $^{14}\text{CO}_2$ .

The alcoholic supernatant (see text) was evaporated to dryness. Chlorophyll was extracted from the solids by washing twice with 0.5 ml chloroform. After evaporating off excess chloroform the solids were taken up in 0.25 ml methanol, and 50  $\mu\text{l}$  of this subjected to high voltage electrophoretic separation (see text). Compounds containing  $^{14}\text{C}$  were identified by exposure to photographic film placed in contact with the electrophoretogram for 4 weeks. Areas containing  $^{14}\text{C}$  were punched from the paper and the radioactivity determined by liquid scintillation counting (efficiency 80%).

●-● sucrose; ○-○ malate; ▲-▲ hexose phosphate.

those obtained by Hatch (11) for photosynthesising maize and sugar cane leaves in which malate was also the initial product of carbon dioxide fixation. Thus, while the barley pericarp may not have the anatomical and physiological properties thought to be characteristic of  $\text{C}_4$  plants it is capable of  $\text{C}_4$  photosynthesis.

The role played by the pericarp in the deposition of grain carbohydrate is not clear. In particular the origin of the carbon dioxide fixed is unknown. The transparent layer of tissue surrounding the green layer of pericarp does not appear to have stomata. Thus, it may be impermeable to atmospheric carbon dioxide. It is possible, therefore, that the function of the pericarp is to prevent respiratory losses from the endosperm by refixing respired carbon dioxide. A similar function has been suggested for the PEPC of the testa of developing peas (12). At this period in endosperm development

metabolic activity is high (13) and carbon dioxide losses in the absence of PEPC could be considerable. Certainly evidence is available (12) to support the theory that in  $C_3$  plants PEPC activity may be induced during periods when the rate of respiration exceeds the rate of photosynthesis. Selection of plants with an efficient carbon dioxide trapping mechanism could form the basis of a successful breeding programme.

In conclusion we have shown that the photosynthetic properties of the barley pericarp are quite different from those of the leaves. The first formed product of photosynthesis is the  $C_4$  acid malate which is then rapidly converted to sucrose. It seems likely that this is a result of the unique environment of the pericarp, situated as it is, between an impermeable transparent layer and the rapidly respiring endosperm. Thus, barley embodies some of the features characteristic of  $C_4$  or tropical plants.

ACKNOWLEDGMENT: We thank the Agricultural Research Council for support.

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## CARBOHYDRATE OXIDATION IN DEVELOPING BARLEY ENDOSPERM

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## SUMMARY

The presence of a number of pentose phosphate pathway (PPP) enzymes in developing barley endosperm is reported. The relative importance of the Embden-Meyerhof-Parnas pathway (EMPP) and the PPP throughout endosperm development was assessed by following the changes in activity of five EMPP and four PPP enzymes. The patterns of activity of the EMPP enzymes were very similar with pronounced peaks of activity at 35 days after anthesis thereafter falling to low or zero levels at the onset of maturity. The PPP enzymes, on the other hand, rose to maximum levels around 30–35 days falling only slightly thereafter.

## INTRODUCTION

Development in cereal grains is characterized by a net synthesis and deposition of storage materials such as starch and protein. Simultaneously, however, catabolic processes may degrade these macromolecules. For example, starch-hydrolysing enzymes such as  $\alpha$ - and  $\beta$ -amylases and phosphorylase are present in developing barley grains (Bilderback, 1971; Duffus and Rosie, 1973). Glycolytic and mitochondrial enzyme activity is also present (Duffus, 1970; Baxter and Duffus, 1973). In addition, electron micrographs of developing barley endosperm show large numbers of mitochondria (Buttrose, 1960), many of which are grouped around the immature amyloplasts (Williams, 1976). This evidence suggests that active respiratory processes accompany grain development and supply energy for starch and protein synthesis.

While some measurements have been made of enzyme activity of the Embden-Meyerhof-Parnas pathway (EMPP) in developing cereal endosperm (Baxter and Duffus, 1973), little is known of the contribution, if any, made by the enzymes of the pentose phosphate pathway (PPP) or their relationship to those of the EMPP. Gibbs and Beevers (1955) studied changes which occur in the relative capacities and activities of the EMPP and the PPP during root differentiation. In apical tissues, oxidation was mainly via glycolysis but, as the cells began to elongate and vacuolate, the PPP activity increased. A similar approach was used by Fowler (1971) when studying carbohydrate oxidation during the growth of cells of sycamore (*Acer pseudoplatanus* L.) in suspension culture. In the early stages, there was a relatively greater increase in the activity of the PPP enzymes compared to those of the EMPP. At later stages of growth, however, little difference was seen in the activities of key enzymes of the two pathways, although labelling experiments using the release of  $^{14}\text{CO}_2$  from (1- $^{14}\text{C}$ )- and (6- $^{14}\text{C}$ )-glucose indicated that carbohydrate oxidation may be mainly *via* the EMPP.

A major function of the PPP may be to supply NADPH for reductive processes or pentose sugars for nucleotide synthesis, whereas the EMPP in conjunction with the tricarboxylic acid

cycle (TCAC) supplies ATP and carbon precursors for general biosynthetic processes. Thus, a study of the relative importance of the EMPP and PPP throughout development in barley endosperm may provide information on the changing metabolic status of the cells.

This paper demonstrates the presence of four PPP enzymes in immature barley endosperm and compares their activities throughout development with those of five enzymes of the EMPP.

## MATERIALS AND METHODS

*Plant material.* The two-row barley, *Hordeum distichum* (L). Lam. cv. Julia, was used throughout. Conditions of growth and methods used to determine the date of anthesis were as described by Merritt and Walker (1969).

*Preparation of endosperm extracts.* The husk, testa-pericarp and embryo were removed by hand and the remaining intact endosperm (including the aleurone layer) used as starting material. As soon as possible after extraction, the endosperms were resuspended in the appropriate buffer at 4°C and homogenized by hand in an all-glass homogenizer. After filtration through two layers of muslin to remove cell debris, the suspension was centrifuged at 4°C for 1 h at 75,000 (Rav) in a swing-out rotor. The supernatant was used as the source of enzyme in all assays. The number of endosperms used for extraction varied from sixty (at 5 days after anthesis) to ten (at 50–60 days after anthesis) per 6 ml buffer. The buffer to be used in the subsequent enzyme assay system was used as the extraction medium in each case.

*Enzyme assays.* All enzyme assays were coupled to oxidation or reduction of NADP which was determined at 340 nm and 30°C in a Unicam SP 800 double beam recording spectrophotometer. Standard assays were used although, when necessary, conditions were modified to give maximum rates. Initial reaction rates were used. Enzymes assayed were phosphofructokinase (EC 2.7.1.11) (Dennis and Coultate, 1967), hexokinase (EC 2.7.1.1) (Tsai, Salamini and Nelson, 1970), pyruvate kinase (EC 2.7.1.40) (Hess and Wieker, 1974), fructose-1, 6-biphosphate aldolase (EC 4.1.2.13) (Rutter *et al.*, 1966), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Glock and McLean, 1953), phosphoglycerate kinase (EC 2.7.2.3) (Bergmeyer, Gawehn and Grassl, 1974), 6-phosphogluconate dehydrogenase (EC 1.1.1.44) (Glock and McLean, 1953), transketolase (EC 2.2.2.1.1) (De la Haba and Racker, 1955) and transaldolase (EC 2.2.1.2) (Venkataraman and Racker, 1961). With pyruvate kinase, hexokinase, phosphoglycerate kinase and phosphofructokinase, the buffer used was 0.05 M HEPES (N-2-hydroxyethyl piperazine-N-ethane sulphonic acid), pH 7.5. With transaldolase, assay V of Venkataraman and Racker (1961) was used in which fructose-6 phosphate and erythrose-4-phosphate combine in the reverse direction to form glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate. In the case of transketolase, xylulose-5-phosphate and ribose-5-phosphate were used as substrates.

With the exception of phosphofructokinase the mean standard deviation of all results was 15%. Considerable variation was experienced in phosphofructokinase extraction and the mean standard deviation was 25%. Therefore, trends in activity rather than individual values were subsequently compared.

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## RESULTS

Fig. 1. shows the changes in activity, throughout barley endosperm maturation, of five EMPP enzymes. The overall pattern of activity is very similar. All have a pronounced peak of activity close to 35 days after anthesis and levels of activity fall rapidly to low or zero values by 55-60 days after anthesis. Only hexokinase and 3-phosphoglycerate kinase were detected at 5 days after anthesis. By 10 days, fructose-1, 6-bisphosphate aldolase had appeared followed at 15 days by phosphofructokinase and, at 20-25 days, by pyruvate kinase.

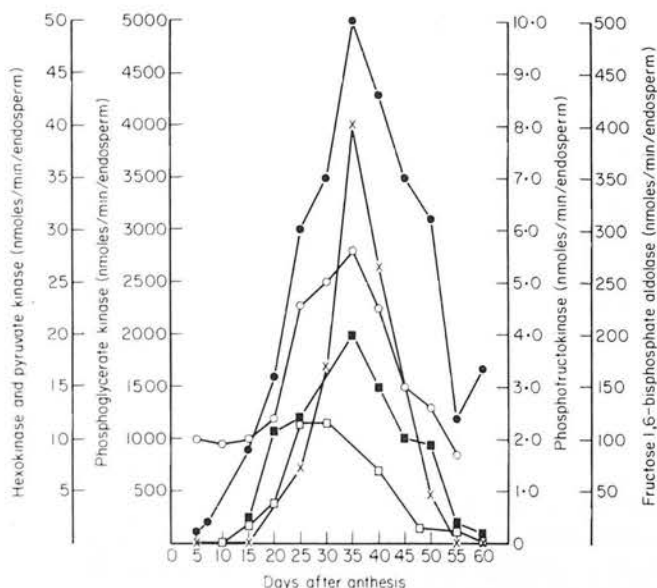


Fig. 1. Changes in activity of enzymes of the EMPP during barley endosperm development. (○) Phosphofructokinase; (○) hexokinase; (X) pyruvate kinase; (■) fructose-1,6 biphosphate aldolase; (●) phosphoglycerate kinase.

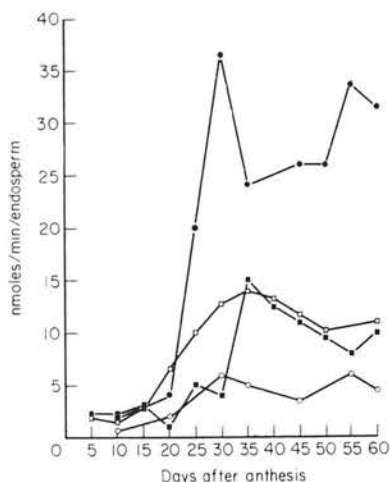


Fig. 2. Changes in activity of enzymes of the PPP during barley endosperm development. (○) Glucose-6-phosphate dehydrogenase; (●) 6-phosphogluconate dehydrogenase; (□) transketolase; (■) transaldolase.

Specific activities varied greatly. The lowest activity was that of phosphofructokinase with a maximum value of 2.3 nmoles/min/endosperm and the highest 3-phosphoglycerate kinase with a maximum value of 5000 nmoles/min/endosperm.

Similarly Fig. 2 shows the time course of activity of four PPP enzymes. In this case also, the overall pattern for each enzyme is similar. All four enzymes were detectable from 10 days after anthesis. Activity then rose, slowly at first, to a maximum value around 30-35 days after anthesis, thereafter remaining fairly constant or falling slightly. Specific activities fell within the narrow range, zero to 36 nmoles/min/endosperm.

## DISCUSSION

There are significant differences in the patterns of activity of EMPP and PPP enzymes during endosperm development. The most notable is that the EMPP enzymes peak at 35 days and have relatively low values at the beginning and end of maturation whereas the PPP enzymes plateau around 35 days and decrease relatively little thereafter. While the factors controlling the activities of the two pathways are complex, the retention of PPP enzyme activity in the later stages of maturity may simply reflect a relatively lower sensitivity to dehydration—a process which begins about 20-25 days after anthesis (Baxter and Duffus, 1973). It seems unlikely that the function of such PPP activity is solely to provide NADPH for lipid biosynthesis in the later stages of endosperm development since this is relatively low at this period compared to that in the first week of development when nuclei, mitochondria, proplastids, endoplasmic reticulum and Golgi bodies first appear (Buttrose, 1960; Williams, 1976). The lipid content of mature barley endosperm is less than 1%. More probably, therefore, the PPP is a source of pentose phosphates for nucleic acid and nucleotide biosynthesis. Certainly at this time, RNA is accumulating rapidly in both the endosperm (Duffus, unpublished) and embryo (Duffus and Rosie, 1975). The technique for estimating relative activities using the release of  $^{14}\text{CO}_2$  from added ( $1\text{-}^{14}\text{C}$ ) and ( $6\text{-}^{14}\text{C}$ ) glucose cannot easily be used in this system since both polysaccharides and their degradation products together with such hydrolytic enzymes as amylases and phosphorylases (Bilderback, 1971; Duffus and Rosie, 1973) are present in the tissue. Because of this, neither steady state conditions nor estimates of glucose utilized are easily obtained. In any case, such information would give an indication only of the relative capacities of the two pathways. Their relative importance in terms of the actual flux of material through each cannot be assessed by this technique.

Phosphofructokinase had the lowest activity of the EMPP enzymes measured and 6-phosphogluconate dehydrogenase the lowest activity of the PPP enzymes. Similar results were reported by Fowler (1971) for cells of sycamore in culture. This suggests that in these as well as in other tissues (Fowler and ap Rees, 1970; Saggerson and Greenbaum, 1969), the two pathways may be regulated at the level of these enzymes. Since, by 48 days, phosphofructokinase had fallen to an extremely low level, it is possible that the functions of the EMPP are supplemented or replaced at this time by the PPP. The relatively high initial levels of hexokinase may reflect its additional role in the conversion of fructose to glucose-1-phosphate via hexokinase, glucose-6-phosphate ketoisomerase (EC 5.3.1.9) and phosphoglucomutase (EC 2.7.5.1). Fructose is derived from sucrose entering the endosperm by the action of sucrose-UDP glucosyl transferase and can subsequently be converted to starch via glucose-1-phosphate and starch phosphorylase (EC 2.4.1.1) or via pyrophosphorylase (EC 2.7.7.9) and starch synthetase (EC 2.4.1.11) (Baxter and Duffus, 1973).



The results reported here, however, while indicating the presence of certain enzymes of the two pathways in developing endosperm together with an estimate of their relative activities under the conditions of assay employed, do not give a quantitative estimate of the relative carbon flow through the pathways. Until *in vivo* measurements of glucose turnover and relative concentrations of its metabolic products and cofactors are available, knowledge of the regulation of hexose metabolism in developing endosperm will remain on a qualitative basis.

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# Separation and Some Properties of Large and Small Amyloplasts throughout Development in Barley Endosperm

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## ABSTRACT

A method is described whereby amyloplasts from immature barley (*Hordeum distichum* L.) endosperm could be separated into two populations of large and small amyloplasts at all stages of development. The small amyloplasts had more amylopectin than the large at early stages, but by 60 days after anthesis, the large had the greater proportion of amylopectin. Starch synthetase activity was associated with both types of amyloplast. The nucleotide specificity of the starch synthetase associated with each population varied independently throughout development. At 25 days after anthesis, the large amyloplasts were more susceptible than the small to  $\alpha$ -amylolysis; however, at 38 and 60 days, the small amyloplasts became more susceptible.

Starch is stored in plants in organelles called amyloplasts, which are deposits of starch, possibly incorporating some of the enzymes of starch synthesis (7), and which may be surrounded by a membrane (5). In barley endosperm, there is generally only one starch granule in each mature amyloplast. Amyloplasts in barley can be divided into two populations—large and small. The initial, transparent, bean-shaped granules which can be seen in the amyloplasts of barley endosperm at 4 days after anthesis grow rapidly and may be up to 35  $\mu$ m in diameter at maturity. At about 14 days after anthesis, small, spherical amyloplasts appear. These do not grow larger than 10  $\mu$ m in diameter. May and Buttrose (16) have called the large granules type A and the small ones type B. Type A granules were said to be initiated up to 2 weeks after anthesis but not later, whereas type B granules are produced only after 14 days. No evidence, however, was presented to support this. Buttrose (6), in his study of developing wheat amyloplasts from immature endosperm, stated that in each plastid there occurs the initiation of one starch granule which grows rapidly in size. This is followed later by the initiation of small amyloplasts in the stroma space. The ratio by numbers of large and small amyloplasts in different barley varieties at maturity has been found to vary from 5.5:1 to 35:1 (14). There are conflicting results concerning the amylose and amylopectin contents of large and small amyloplasts. Bathgate and Palmer (1) have reported the amylose content of small barley amyloplasts to be higher than that of the large; conversely, Waring and De Haas (13) have shown that the large have a higher amylose content than the small. Other results have shown no significant difference (11). Since some discrepancies observed may be due to the techniques of separation, a method was required which ensured satisfactory separation of amyloplasts. Methods using Ludox and Urografin density gradients have been found to be successful in the separation of large and small potato amyloplasts (9). These did not prove successful with immature

barley amyloplasts probably because these are much smaller than potato amyloplasts. A method was therefore devised to separate the two populations of barley amyloplasts and some of their properties are described.

## MATERIALS AND METHODS

**Plant Material.** Barley (*Hordeum distichum* [L.] Lam. var. Julia) was used throughout. The age of the barley (in days after anthesis) was determined subjectively with reference to the table of Baxter (3) based on the method of Merritt and Walker (17).

**Separation of Large and Small Amyloplasts.** Five endosperms 25 days after anthesis were homogenized in 1 ml 0.15 M K-phosphate buffer, pH 7.3, at 4 C in a Potter-type all glass handheld homogenizer. The homogenate was filtered through two layers of muslin and 0.5-ml filtrate layered on top of a discontinuous sucrose gradient consisting of 1 ml each of 80%, 60%, 40%, and 20% (w/v) sucrose, in a 5-ml cellulose-nitrate tube. This was then centrifuged at 500g, 4 C for 10 min in a swing out head. After centrifugation, the original layers which were still visible were separately removed with a Pasteur pipette and the pellet at the bottom was scraped off and resuspended in buffer. The amyloplasts could be recovered from the sucrose solutions by centrifugation. When larger quantities were required, 50 endosperms were homogenized in 10 ml buffer. After filtration as before, the filtrate was layered on a gradient of 20 ml each of 80%, 60%, 40%, and 20% sucrose in a 100-ml centrifuge tube. This was centrifuged and the layers removed as for the smaller gradient.

The 60% layer was used as a source of small amyloplasts and the pellet at the bottom as a source of large amyloplasts.

The method for counting amyloplasts was adapted from May and Buttrose (16).

**Determination of Amylopectin and Amylose Content of Amyloplasts.** Large and small amyloplasts were prepared on the sucrose gradient and washed three times with distilled H<sub>2</sub>O. They were then dried at 40 C for at least 48 hr. Amylopectin and amylose contents were determined using the blue value method of Gilbert and Spragg (12), except that 1 mg amyloplasts was used and reagent quantities were adjusted accordingly.

**Starch Synthetase.** Starch synthetase activity was estimated in large and small amyloplast fractions by a colorimetric assay with either ADPG or UDPG as the nucleotide sugar substrate. The amyloplasts were suspended in 1 ml glycine-NaOH buffer, pH 8.6, and 0.2 ml used for the assay. After incubation at 25 C for 2 hr, the ADP or UDP produced was estimated by the method of Leloir (15).

**Degradation of Amyloplasts by  $\alpha$ -Amylase.** Five mg washed and dried amyloplasts were suspended in 1.9 ml HEPES buffer 6.9, except for one experiment with amyloplasts from 25-day barley when 2 mg were used. To this was added 0.1 ml  $\alpha$ -amylase (0.01 unit) from hog pancreas. The reagents were

incubated at 25 C. One-tenth ml buffer replaced the enzyme in the control experiment. Samples (0.01 ml) were removed at known time intervals and their reducing sugar content estimated by the method of Somogyi (20) as modified by Nelson (18).

**Amyloplast Degradation at Germination.** Endosperms were dissected out from barley at two ages of developing grain and at four stages of germination. These were homogenized in 1 ml iodine solution (0.2%  $I_2$  in 2% KI) and the numbers of large and small amyloplasts determined.

## RESULTS

**Separation of Large and Small Amyloplasts.** The 60% sucrose layer was found to contain an almost homogeneous population of small amyloplasts and the pellet at the bottom of the gradient was found to contain a similarly homogeneous population of large amyloplasts. Light micrographs of the original homogenate and large and small amyloplast fractions are shown in Figures 1, 2, and 3, respectively. The 80% sucrose layer contained a mixture of large and small amyloplasts. The 20% and 40% sucrose layers contained small amyloplasts, mixed with other small particles and larger portions of intact cells. Most of these larger pieces from the original homogenate were removed by the initial filtration through double muslin. Sixty-seven per cent of the amyloplasts from the homogenate were recovered from the gradient. The ratio of small to large that were recovered from barley 25 days after anthesis (3.5:1) was almost exactly the same as the ratio found *in vivo* at that age (3.9:1). Electron micro-

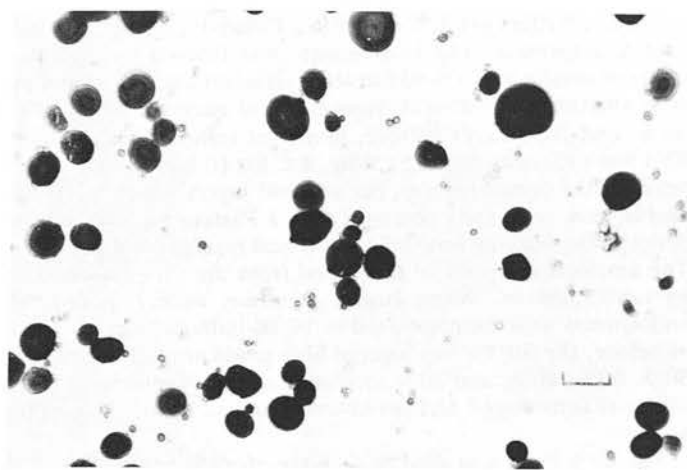


FIG. 1. Whole endosperm homogenate before separation on the sucrose gradient. Bar marker = 20  $\mu$ m.

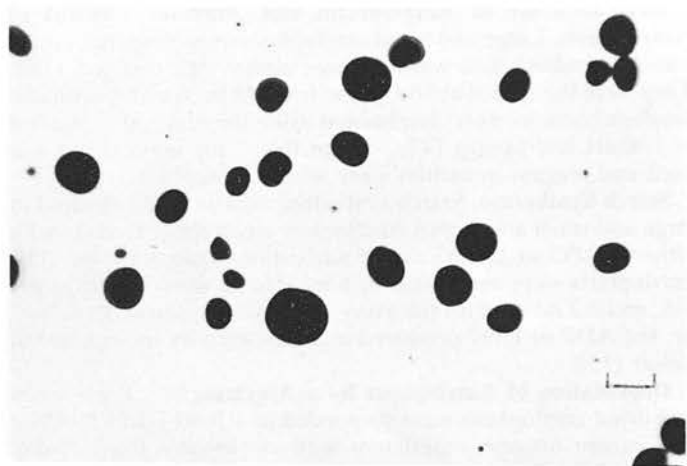


FIG. 2. Large amyloplasts after separation on the gradient. Bar marker = 20  $\mu$ m.

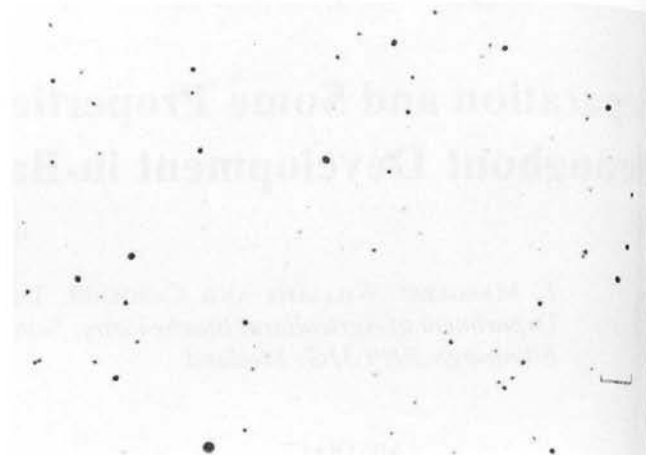


FIG. 3. Small amyloplasts after separation on the gradient. Bar marker = 20  $\mu$ m.

graphs did not show that any damage had been caused to the amyloplasts by the techniques used. The large amyloplasts are ellipsoid and, at 25 days after anthesis, measure 16 to 20  $\mu$ m by 12 to 16  $\mu$ m. The small are spherical, 4 to 6  $\mu$ m in diameter at 25 days after anthesis.

**Determination of Amylopectin and Amylose Content.** The amylopectin and amylose contents of the separated large and small amyloplasts from 25 days after anthesis are shown in Figure 4. Initially, the large amyloplasts contain 75% amylopectin. This falls to 55% amylopectin at 40 days after anthesis, and then rises again to 71% by 60 days after anthesis. At 25 days after anthesis, the amylopectin content of the small amyloplasts is 95%. This drops to 69% at 35 days after anthesis and remains around this level until 50 days after anthesis. After this, it drops again, until by 60 days after anthesis, the amylopectin content is only 60%. This means that the amylopectin content of the small amyloplasts is higher than that of the large amyloplasts throughout development until 60 days after anthesis when it falls below that of the large amyloplasts.

**Starch Synthetase.** The starch synthetase associated with large amyloplasts was found to be active with UDPG only between 22 and 25 days after anthesis (Fig. 5). Activity with ADPG was present by 22 days after anthesis and continued throughout development. The maximum activity with ADPG occurred at 40 days after anthesis. With the small amyloplasts, the opposite effect was seen (Fig. 5B). Until 22 days after anthesis, the starch synthetase was active with ADPG; however, after this age, it was active with UDPG.

**Degradation of Amyloplasts by  $\alpha$ -Amylase.** At 25 days after anthesis, the large amyloplasts were more susceptible to amylolysis (Fig. 6); however, at 38 and 60 days, the small amyloplasts became more susceptible (Fig. 7). Thus, the rate of reducing sugar production from amyloplasts does not appear to depend upon the percentage amylopectin content of the starch in those amyloplasts.

**Amyloplast Degradation at Germination.** The ratios of small to large amyloplasts at different stages in development at germination are shown in Table I.

The number of small amyloplasts compared to the number of large increases during development. At germination, there is a slight but insignificant drop in the ratio, but this increases again as the shoot develops, with a very rapid increase between the 10-cm and 15-cm shoot stage.

## DISCUSSION

The amyloplast separation technique described here has several advantages. We found that the same ratio of small to large amyloplasts was observed before and after separation, and

% AMYLOPECTIN

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10<sup>3</sup> AMYLOPLASTS

UDPG OR ADP PRODUCED ( $\mu$ mole)

10 5 0 5 10 15

0 5 10 15

FIG. 5. Star  
slight but insignificant drop in the ratio, but this increases again as the shoot develops, with a very rapid increase between the 10-cm and 15-cm shoot stage.

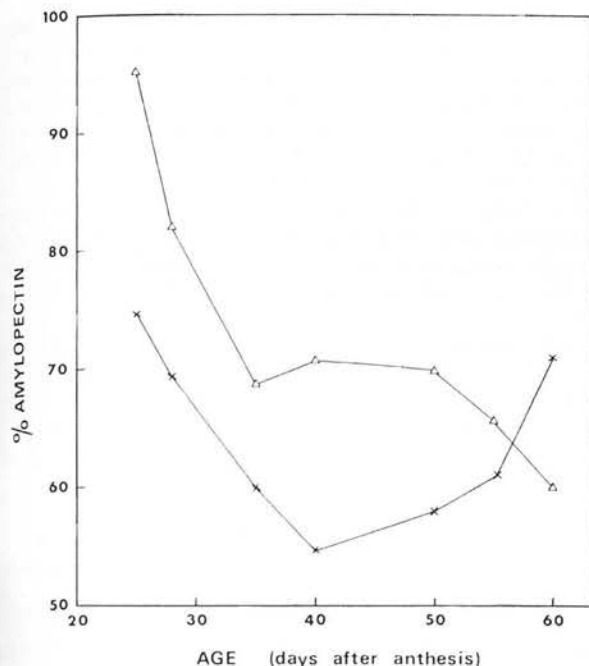


Fig. 4. Percentage amylopectin content of large and small amyloplasts throughout development. Large amyloplasts (x); small amyloplasts (Δ). Mean SD =  $\pm 4.5$ .

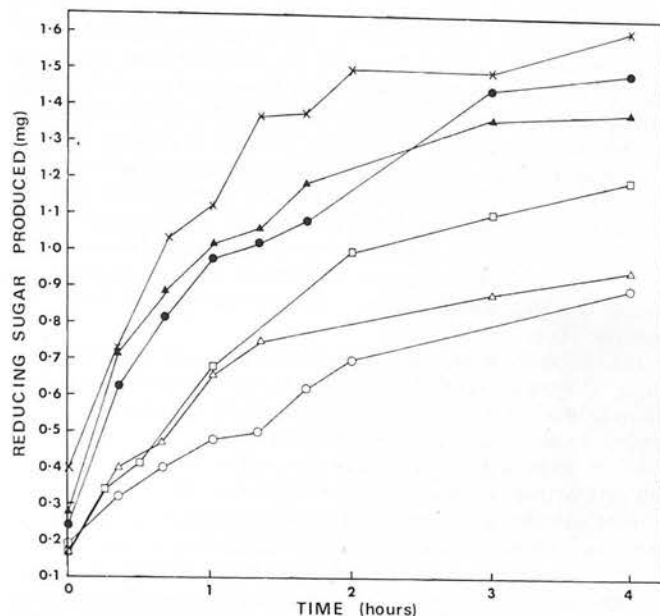


Fig. 6.  $\alpha$ -Amylase degradation of large and small amyloplasts. Small amyloplasts, germinated grain (x); small amyloplasts 60 days after anthesis (Δ); small amyloplasts, 38 days after anthesis (●); large amyloplasts, germinated grain (○); large amyloplasts, 60 days after anthesis (Δ); and large amyloplasts, 38 days after anthesis (□).

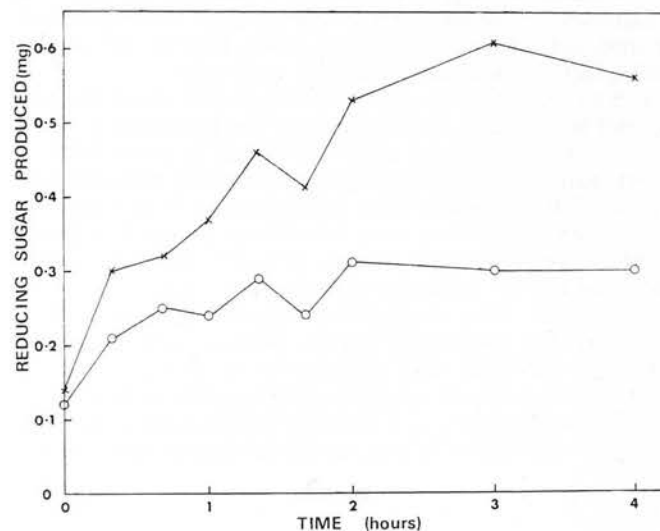


Fig. 7.  $\alpha$ -Amylase degradation of large and small amyloplasts from endosperms 25 days after anthesis. Large amyloplasts (x); small amyloplasts (○).

Table 1. Ratio of Small:Large Amyloplasts at Different Stages in Development and Germination

Stage in development or germination	Ratio small:large (average of at least 3 determinations) Mean s.d. = $\pm 3.3$
25 days after anthesis	3.9:1
60 days after anthesis	9.2:1
Germinated-radicle just showing	8.2:1
0.7 cm shoot	16.5:1
5 cm shoot	25.1:1
15 cm shoot	133.0:1

Starch synthetase activity in large and small amyloplasts throughout development. A: Starch synthetase activity in large amyloplasts (x); activity with ADPG (Δ). Mean SD =  $\pm 2.2$ .

Structure of the amyloplasts was preserved, as seen under the microscope. At least some of the associated enzymic activity was retained. Amyloplasts were separated on the sucrose gradient, and differences in amylose and amylopectin contents between large

found in the amylopectin contents of large and small in normal Glacier. The situation is reversed in Julia in the stages of development leading up to maturity when the small amyloplasts have a higher percentage amylopectin content than the large. One explanation for these results could be that there are, in fact, two types of small amyloplast. The first type (type A) would be produced at the start of development and would be high in amylopectin, accounting for the high amylopectin content of amyloplasts early in development. These would grow to become large amyloplasts. They are really no different from the large amyloplasts and are more accurately described as young large amyloplasts. The other type (type B) of small amyloplasts would be produced later in development—possibly at around 14 days after anthesis when the two populations of amyloplasts are first seen. These would have a higher amylose content than type A. Because they would stay small throughout development, they would account for the majority of small amyloplasts at maturity, and hence the higher amylose content of small amyloplasts at that time. This would confirm the observation of May and Buttrose (16).

In addition to the developmental differences of starch composition, the starch synthetase of the separated amyloplasts has differing nucleotide specificities. The amyloplast-bound starch synthetase of barley endosperm, older than 12 days after anthesis, is active with both UDPG and ADPG as glucose donors although activity is greater with ADPG (4). The different nucleotide specificities of large and small amyloplast starch synthetases may be accounted for by the relative activities of the enzymes associated with the large and small amyloplasts. If the majority of the small amyloplasts seen at early ages are going to develop into large amyloplasts, it is not surprising that small amyloplasts at this stage are active with ADPG. The activity with UDPG might, therefore, be due to the second type of small amyloplasts. The early activity of the large amyloplasts with UDPG is more difficult to explain. It is possible that the starch synthetase of the large amyloplasts is capable of utilizing both nucleotides until about 22 days. After this time, the enzyme may be modified and loses its ability to utilize UDPG. The activity with UDPG is then seen in the small amyloplasts.

Dunn (10) has shown that bacterial or malt  $\alpha$ -amylase will degrade maize starch granules *in vitro*. This is confirmed by the present work. No definite correlation could be made between either amyloplast size or amylopectin to amylose ratio and ease of  $\alpha$ -amylolysis since the large amyloplasts at 25 days and the small at 38 and 60 days were more easily degraded. Furthermore, during germination, the ratio of small to large amyloplasts increased rapidly, indicating that the large amyloplasts were degraded first. These results, taken together, indicate that factors other than composition and size influence susceptibility to attack by  $\alpha$ -amylase. This is borne out by previous work by Palmer (19) who found, using the scanning electron microscope, that the small granules were degraded first on malting, whereas Bathgate and Palmer (2), using malt  $\alpha$ -amylase on barley starch,

found that the small amyloplasts were more resistant than the large. In this context, extracts from germinated barley did not appear to degrade immature amyloplasts. Thus, the membrane structure of the immature organelle may be less permeable to barley amylases than hog pancreatic  $\alpha$ -amylase. This is likely since the immature amyloplast must have resistance to the hydrolases known to be present in the developing grain (8).

The activity, source, and purity of the hydrolytic enzymes used, together with the permeability properties of the amyloplast membrane (when present), must be considered when devising experiments to study amyloplast structure by degradative techniques.

It is clear that there are at least two populations in developing barley endosperm. They differ in size, composition, and associated enzyme activity. Further work in this laboratory using  $^{14}\text{C}$  labeling has confirmed that those amyloplasts produced just after anthesis grow to become large amyloplasts. The small amyloplasts are produced later in development.

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## **The *In Vitro* Culture of Immature Barley Embryos on Different Culture Media**

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## The *In Vitro* Culture of Immature Barley Embryos on Different Culture Media

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### ABSTRACT

Immature barley embryos (*Hordeum distichum* var. Julia) of between 0.20 and 0.80 mm in length, were isolated from the developing grain and cultured *in vitro* on various culture media. The subsequent development of the embryos was followed over a period of weeks, and where germination ensued the growth rate of shoot and root meristems was compared with *in vivo* germination rates. Various growth media were assessed for their ability to support normal development of immature embryos. A number of published media failed to support satisfactory development of young embryos. The addition of 1-15 per cent coconut milk to Norstog's Medium I (mineral + vitamin solns) enhanced embryo development and lowered the threshold of viability from embryos of 0.50 mm in length to 0.35 mm. Although in many cases germination ensued, embryo development was largely abnormal. A slightly greater enhancement of growth was achieved with 0.05-0.30 per cent casein hydrolysate as the growth medium supplement, although abnormal development was not eliminated. A further lowering of the viability threshold to include embryos of 0.25 mm in length was obtained by combining 2.7 mM glutamine with the casein hydrolysate supplement. Normal development and germination of embryos as small as 0.25 mm was however obtained on Norstog's Medium II and the results were reproduced in four additional *H. distichum* varieties. In each case the critical threshold of viability was found to lie in embryos of 0.20-0.30 mm in length.

### INTRODUCTION

The *in vitro* culture of plant embryos has been a technique of interest to scientists for many years and reports as early as 1924, by Dietrich, describe the growth and germination of excised Gramineae embryos. However, only in recent years has particular import been placed on the successful culture of Gramineae embryos, owing to new developments in plant breeding. This applies in particular to the interspecific hybrids created in crosses such as rye  $\times$  barley (Kruse, 1969) and the haploid embryos obtained from crossing *H. vulgare* with *H. Bulbosum*, (Kasha and Kao, 1970). The incompatibility which develops between embryo and endosperm in seeds derived from many hybrid crosses, necessitates their isolation and culture in order to ensure seedling formation.

One of the first detailed comparisons of *in vivo/in vitro* barley embryo development was published by Merry in 1942. Embryos were grown on solid media containing diluted Shrive's solution R5S2 and 2 per cent sucrose. Various thresholds of growth potential were noted, namely a complete failure of embryos smaller than 0.35 mm to develop, and only limited growth of embryos between 0.35 and 0.60 mm long. Embryos of 0.75 mm and above, grew and germinated, but their growth pattern failed to reproduce accurately that observed *in vivo*.

Numerous attempts have been made to improve the viability of small embryos and to design growth conditions which allow the *in vivo* growth pattern to be reproduced *in vitro*.

Various undefined growth supplements have been included in the culture media such as coconut milk, barley endosperm extract and casein hydrolysate, but results have been conflicting (Chang, 1963; Davies, 1960; Kruse, 1974; Norstog, 1961; Ziebur and Brink, 1951). Norstog and Smith (1963) published details of a new growth medium comprising a modified White's mineral medium combined with a mixture of certain amino acids, organic acids, vitamins and sucrose. The authors claimed that the growth and differentiation of embryos  $>0.20$  mm was identical with those *in vivo*, and that embryos as small as  $0.01$  mm could be induced to form callus tissue. Further modifications to the mineral and organic components of the medium were published in 1973 (Norstog), enabling a higher survival and growth rate to be obtained with  $>0.20$  mm embryos.

At first sight the achievements of these research workers (Norstog, 1973; Kasha and Kao, 1970) indicates that all the problems in barley embryo culture are solved and that the system is ripe for the *in vitro* investigations of embryo growth by the developmental biologist. However, initial attempts to repeat several of the published results indicated that many of the media were unreliable and thus unlikely to be of any value, particularly in a large-scale barley breeding programme.

This paper reports the results of a survey of various published culture media, combined with a number of the authors' own modifications, assessing the conservation of *in vivo* growth pattern in cultured embryos and determining the threshold of viability. The comparative morphology of germinating embryos grown *in vivo* and *in vitro*, was used as a sensitive indicator of abnormal development in cultured embryos. Some consideration is given to the nature of the natural environment of the developing embryo and the importance of reproducing this in the artificial culture system.

#### MATERIALS AND METHODS

The two-row barley *Hordeum distichum* (L.) Lam. cv. Julia was grown in a greenhouse, at approximately  $20^{\circ}\text{C}$ , under natural light, extending the daylength to 18 h with mercury vapour lamps. *H. distichum* (L.) cv. Abacus, Golden Promise, Hassan, Imber and Julia, were also grown in the field and sampled in the summer of 1976. The first six tillers of any plant were labelled at the time of anthesis, as described by Merritt and Walker (1969), and the ears removed at various stages of their development. The grains, from the central portion of each ear, were dehusked and sterilized in five per cent sodium hypochlorite for 15 minutes, and thoroughly rinsed in sterile distilled water. The embryos were excised in sterile conditions, in a droplet of six per cent sucrose solution, and transferred to culture media with the aid of a dissecting microscope.

The culture media were prepared as prescribed by their authors, and the composition of the media and the various modifications is given in Table 1. Separately autoclaved sucrose, in addition to seitz-filtered amino acid and vitamin solutions, were added to the mineral-molten agar mixture and poured into sterile petri dishes and capped test tubes ( $100 \times 16$  mm). The coconut milk and casein hydrolysate ingredients were autoclaved.

High sucrose concentrations in culture media have been used to control precocious germination and promote embryo development. These observations led to the transfer of embryos from nine per cent sucrose media to six per cent and three per cent sucrose media after nine and 13 days of culture respectively.

The excised embryos were placed on media in petri dishes to facilitate microscopic growth measurements, with an ocular micrometer, but were transferred to test tubes containing 5 ml media at the onset of germination. The petri dishes were incubated in the dark at  $20^{\circ}\text{C}$  and the germinating embryos were transferred to an illuminated growth cabinet maintained at  $20^{\circ}\text{C}$  with an 18 h day and 8000 lx illumination.



TABLE 1. Composition of growth media for embryo culture

Components mg l <sup>-1</sup>	Norstog's Medium*		Kasha's Medium§		Norstog's Medium		0.1 per cent casein   hydrolysate
	I†	II‡			I	II	
<i>Major elements</i>				<i>L-Amino acids</i> (mmoles/l)			
MgSO <sub>4</sub> ·7H <sub>2</sub> O	730	740	250	Alanine	4.5	0.56	0.32
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	290			Arginine		0.06	0.21
Na <sub>2</sub> SO <sub>4</sub>	200		2500	Asparagine			
KNO <sub>3</sub>	160			Aspartate			0.36
KCL	140	750		Cysteine		0.17	
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	800		150	Cystine			0.01
KH <sub>2</sub> PO <sub>4</sub>		910		Glutamate			1.36
CaCl <sub>2</sub> ·2H <sub>2</sub> O		740	150	Glycine	0.08		0.25
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>			134	Histidine			0.19
<i>Trace elements</i>				Hydroxyproline			
MnSO <sub>4</sub> ·4H <sub>2</sub> O	3	3	16	Isoleucine			0.40
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.5	0.5	3	Leucine	0.15	0.08	0.71
H <sub>3</sub> BO <sub>3</sub>	0.5	0.5	3	Lysine			0.56
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.25	0.025	0.028	Methionine			0.16
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25	0.025	0.250	Phenylalanine	0.06	0.06	0.30
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.25	0.025	0.025	Proline			
Fe-Citrate	10	10		Serine			0.48
Fe-EDTA			28	Threonine			0.29
KI			0.750	Tyrosine	0.06	0.06	0.30
<i>Vitamins</i>				Tryptophan	0.05		0.05
Niacin	1.25		1.00	Valine			0.59
Thiamine-HCl	0.25	0.25	10.00	Glutamine	2.7	2.7	
Pyridoxine-HCl	0.25	0.25	1.00				
Ca-Pantothenate	0.25	0.25					
<i>Additional nutrients</i>							
Malic acid (adjusted to pH 4.9 with NH <sub>4</sub> Cl)	100	1000					
Inositol (meso)		50	100				
Sucrose (per cent)	3-9	3-6	2				
Agar	9000	6000	7000				
Final pH	4.9	5.0	5.5				

## \* Growth medium modifications

## Coconut milk media:

Norstog's Medium I (major and trace elements, vitamins, agar)

+ 1-15 per cent coconut milk

+ 3-9 per cent sucrose.

## Casein hydrolysate media:

Norstog's Medium I (major and trace elements, vitamins, agar)

+ 0.05-0.30 per cent casein hydrolysate (vitamin free)

+ 3-9 per cent sucrose.

Norstog's Medium I (major and trace elements, vitamins, glutamine, agar)

+ 0.10-0.30 per cent casein hydrolysate (vitamin free)

+ 6 per cent sucrose.

† Norstog and Smith (1963).

‡ Norstog (1973).

§ Kasha and Kao (1970).

|| D'Mello and Emmans (1975).

## RESULTS

The suitability of 13 different growth media for embryo culture was assessed with immature embryos varying in size between 0.20-0.80 mm.

*In vivo embryo development*

Since the degree of cellular differentiation may play an important part in determining the growth potential of the excised embryo, the relationship between embryo size and the appearance of distinct morphological markers is given: embryos of up to 0.20 mm in length are non-polar undifferentiated spheres of cells. Between 0.20 and 0.30 mm the embryo becomes club shaped, tapering at the suspensor, and it is up to this stage that this un-

differentiated structure is defined as a proembryo. By 0.35 to 0.40 mm the development of the coleoptile becomes apparent by the appearance of a slight indentation on the embryo face and growth of the stem meristem below it. The development of the scutellum can just be seen in 0.45 mm embryos. More detailed descriptions of developmental embryo morphology are given by Merry (1941) and Chang (1963), and may be referred to for a clearer definition of 'normal' *in vivo* development.

### *In vitro embryo development*

The results of the survey are tabulated in Table 2 and will be described in order of presentation. The measurements of embryo length in Table 2 represents the average of a minimum of ten determinations.

TABLE 2. *The in vitro development of small embryos on various growth media. Small embryos were excised and measured (length) and their subsequent development on each medium classified into five categories according to morphological criteria; the range of embryo sizes (initial) found in each category are shown below*

Growth medium	No growth	Cell enlargement and callus formation	Abnormal development and necrosis or callus formation	Abnormal development and germination	Normal development and germination
Kasha's Medium	0.25-0.40				
Norstog's Medium I					
6% sucrose	0.25-0.45	0.50-0.60			
Norstog's Medium I					
9% sucrose					
+ 1% coconut milk	0.25-0.35	0.35-0.40	0.40-0.55	0.55-0.60	
+ 75% ← ————— → 25%					
5% coconut milk	0.25-0.35	0.35-0.40	0.40-0.45	0.50-0.52	> 0.55
+ 50% ← ————— → 50%					
10% coconut milk	0.25-0.40		0.42-0.50		0.50-0.53
+ 15% coconut milk	0.25-0.45		0.50-0.55		
Norstog's Medium I					
9% sucrose					
+ 0.05% casein hydrolysate	0.25-0.50			0.55-0.65	
0.10% casein hydrolysate	0.25-0.55				
0.30% casein hydrolysate	0.25-0.40	0.40-0.45	0.45-0.50	0.50-0.55	> 0.60
Norstog's Medium I*					
6% sucrose					
+ 0.10% casein hydrolysate		0.25-0.28	0.30-0.40	50% ← ————— → 50%	> 0.55
+ 0.30% casein hydrolysate		0.25-0.35	0.35-0.45	0.40-0.45	> 0.50
				No germination	No germination
		50% ← ————— → 50%			No germination
Norstog's Medium II	0.20-0.25	0.25-0.35			> 0.35
3.42% sucrose					
+ 6% sucrose	0.20-0.25	0.25-0.35			> 0.38

\* + glutamine.

*Kasha's Medium.* This medium, derived from Gamborg's B5 medium failed to support the development of embryos ranging between 0.25 and 0.40 mm. No attempt was made to extend these tests to larger embryos.

*Norstog's Medium I.* Julia embryos smaller than 0.50 mm failed to grow on Norstog's Medium I, and those between 0.50 and 0.60 mm grew mainly by cell enlargement, leading eventually to callus formation (Table 2). Further experiments (not shown) revealed that only in embryos as large as 1.2 mm did embryo growth culminate in germination, and then,

only in a small percentage of the population could the developmental morphology be described as 'normal'. The lack of success obtained with this medium led to an assessment of various modifications to Norstog's Medium I.

*Coconut milk supplemented media.* The components of Norstog's Medium I included in these media are given in Table 1, but in initial experiments ammonium malate was also included. This addition completely blocked the growth of embryos of between 0.25 and 0.60 mm in all four coconut milk media.

From Table 2 it can be seen that coconut milk causes a marked enhancement of embryo growth. In comparison to the complete Norstog Medium I, it can be seen that the threshold of viability on one per cent and five per cent coconut milk media has been lowered to include 0.35 mm embryos. Although their development was abnormal, and their growth rate very slow, embryos of 0.50 and 0.55 mm were found to germinate on 1–10 per cent coconut milk. Normal development was observed in many of the larger embryos (0.55 mm) on both 5 per cent and 10 per cent coconut milk media, although their germination was precocious.

The failure of the smaller embryos to complete certain developmental steps may well account for the eventual formation of callus or cessation of growth.

*Casein hydrolysate supplemented media.* When casein hydrolysate was added to the mineral and vitamin components of Norstog's Medium I, a concentration of 0.3 per cent was required to cause a significant induction of embryo growth (Table 2). However, even at the lower 0.05 per cent concentration, the threshold embryo size at which embryo development led to germination was lowered from 1.2 mm in the complete Norstog medium, to 0.55 mm. A comparison of the two growth supplements, 0.3 per cent casein hydrolysate and five per cent coconut milk, in relation to the threshold embryo size for the expression of growth potential (Table 2), indicates that the coconut milk was more effective. However, a comparison of embryo growth rates and the size of germinating embryos in the two media (not shown), revealed that enhancement of embryo development was greater with casein hydrolysate. Embryos of an initial size of 0.55 mm, cultured on 0.3 per cent casein hydrolysate attained twice the size of those grown on five per cent coconut milk, by the onset of germination. As a result of the normal development and size attained at maturity, the germination of the casein hydrolysate-grown embryos was judged not to be precocious. Although the 0.50 mm embryos also grew faster on 0.3 per cent casein hydrolysate they failed to germinate.

As with the coconut milk media, inclusion of the ammonium malate component of Norstog's Medium I, completely inhibited embryo growth on all casein hydrolysate media.

The addition of glutamine to the 0.3 per cent casein hydrolysate medium, markedly lowered the viability threshold, and increased the growth potential of small embryos. While embryo development had failed to occur on 0.1 per cent casein hydrolysate media, the addition of glutamine induced embryo growth, the rate increasing with the size of the excised embryos (not shown). The final stages of embryo enlargement and germination missing in the glutamine supplemented media, is probably attributable to the constant sucrose concentration. This did not, however, interfere with the expression of normal development in embryos as small as 0.40 mm.

*Norstog's Medium II.* In devising this medium for barley proembryos, Norstog (1973) found that the optimum sucrose concentration was variable, so two concentrations were selected for the present tests. The dramatic improvement over the previous media for *in vitro* embryo culture is immediately clear. 0.45 mm embryos, which are approximately 10 days old, reach maturity after 12 days culture, at which stage a uniform initiation of germination occurs [Fig. 1(a)]. The sucrose concentration of the medium did not appear to be critical, and similar growth rates were obtained for 0.45 mm embryos [Fig. 1(a)].

Unlike the media described previously, the growth potential of the immature embryos is clearly defined in that the embryos are largely either unviable or, at a critical size, capable of normal development. Further, on this medium the threshold embryo size for viability was significantly reduced. Those embryos falling into an intermediate small size class, between 0.20 and 0.30 mm, tended to grow by cell enlargement, and some developed callus tissue. The growth rate of the eight-day-old embryos (0.25 mm) was very similar to the

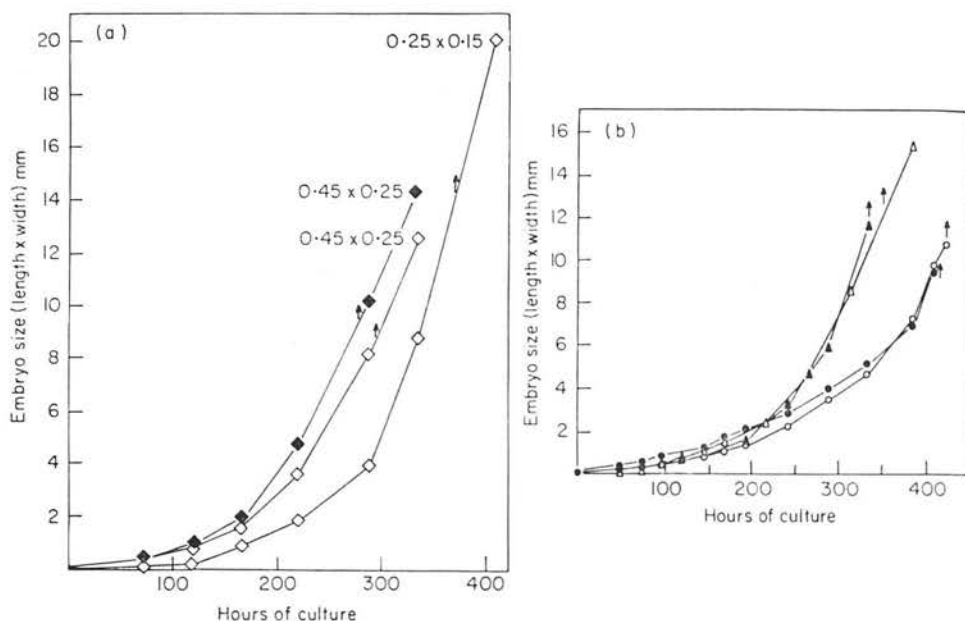


FIG. 1. The growth of small embryos on Norstog's Medium II. (a) Julia embryos of two sizes were grown on Norstog's Medium II containing: ◇, 3.42 per cent sucrose; ◆, 6 per cent sucrose. (b) The following varieties of barley embryo, of various sizes, were grown on Norstog's Medium II: ○, Julia (0.30 x 0.20); ●, Golden Promise (0.45 x 0.30); △, Hassan (0.35 x 0.20); ▲, Imber (0.35 x 0.20).

10-day-old embryos [Fig. 1(a)], development and germination lagging some two–three days behind in the former. Those embryos developing *in vivo*, on plants grown in the greenhouse at 20 °C, only reach maturity some 30 to 35 days after anthesis, at which stage they will immediately germinate when transferred to suitable culture media. From the growth rate of excised embryos shown in Fig. 1(a) it is clear that the development cycle can be artificially accelerated.

In order to eliminate the possibility of varietal differences, the embryo growth of 4 additional varieties (Abacus, Golden Promise, Imber and Hassan) on Norstog Medium II, was determined. Figure 1(b) shows the rapid growth and subsequent germination of all five varieties of barley embryo. Although the embryos of Hassan and Imber developed somewhat faster than the other varieties, some 30–40 per cent of the population failed to develop at all, but this may have resulted from damage during excision. In all varieties the threshold of viability in terms of embryo size was identical, namely 0.20 to 0.30 mm.

The loss of viability in the proembryos might have been attributed to damage during excision, particularly where embryos as small as 0.10 mm are excised. To avoid the possibility of damage, some proembryos were left within the confines of the endosperm tissue, that portion of the endosperm being placed intact on the culture medium. These proembryos similarly failed to develop.

*Germination of in vivo and in vitro grown embryos*

Germination of immature embryos successfully cultured on the different growth media, was compared with embryos excised at maturity (35 days old) and germinated on similar media. Measurements of shoot and root growth of the germinating embryos is given in Fig. 2.

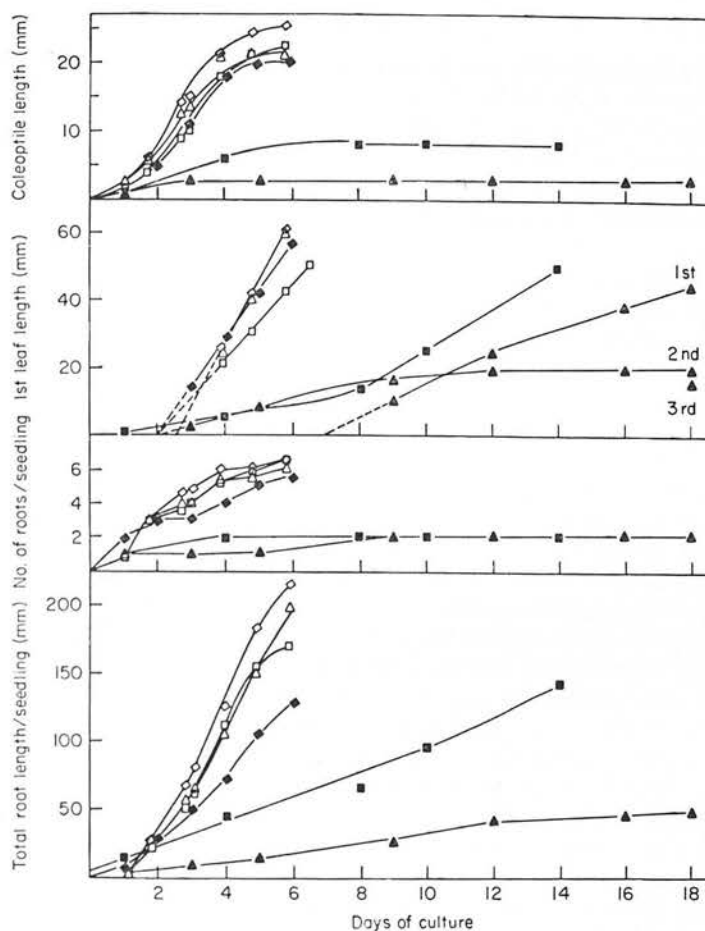


FIG. 2. Germination of *in vivo* and *in vitro* grown 'Julia' embryos. The rate of shoot and root development of excised mature embryos (35 days old), (open symbols); and <0.8 mm cultured embryos, (closed symbols) germinated on the following media:  $\triangle$ ,  $\blacktriangle$ , Norstog's Medium I + 5 per cent coconut milk (3 per cent sucrose);  $\square$ ,  $\blacksquare$ , Norstog's Medium I + 0.3 per cent casein hydrolysate (3 per cent sucrose);  $\diamond$ ,  $\blacklozenge$ , Norstog's Medium II (3.42 per cent sucrose). In the cultured embryos  $T^0$  is taken from the onset of germination.

The conservation of the normal developmental sequence in those embryos grown on Norstog's Medium II was confirmed by the similarity in the germination of *in vivo* and *in vitro* grown embryos.

The germination growth rate of both mature excised embryos and cultured embryos was depressed by the inclusion of 0.3 per cent casein hydrolysate in the media. Although the final growth rates of the 1st leaf in the two sets of embryos was similar, characteristics of abnormal development could be discerned. These included an early cessation of coleoptile growth and poor seminal root growth contributing to a lag in maximum shoot growth.

While superficially those embryos grown on coconut milk media appeared normal, their germination reveals a significant disruption in normal differentiation. Coleoptile growth was severely restricted, and the 1st leaves curled and withered after limited development. These abnormalities of shoot growth were accompanied by slow and delayed root development.

### DISCUSSION

Various published culture media, combined with certain of the authors' own modifications, have been assessed for their use in immature barley embryo culture.

Kasha and Kao (1970) successfully devised a medium for the growth of haploid embryos of *H. vulgare* isolated 10 days after anthesis. Although the survival rates were only about 10 per cent, the low yields could have been attributed to the delicacy of haploid embryos, rather than a deficiency in the medium. In view of the failure of diploid 'Julia' embryos, as large as 0.40 mm, to survive on this medium, this conclusion is unwarranted.

In 1963, Norstog and Smith published a new modified White's medium, which was suitable for the culture of embryos as small as 0.09 mm. Those embryos of >0.20 mm developed in an identical manner to embryos *in vivo*, reaching mature dimensions after only 10 days culture (Norstog, 1965). In the present studies Norstog's Medium I completely failed to support the growth of 'Julia' embryos smaller than 0.50 mm, and in no case was the development of older embryos found to be normal.

Using White's mineral medium and 12 per cent sucrose, Norstog (1961) reported a marked enhancement of embryo growth by 20 per cent coconut milk. Embryos of 0.50 mm attained maturity after 14 days culture, and normal development was achieved in embryos of only 0.37 mm. In addition, viability was attained in embryos measuring 0.125 mm and above. Although coconut milk at an optimum concentration of one to five per cent, enhanced 'Julia' embryo growth on Norstog's Medium I, the viability threshold was only slightly lowered (0.35 mm embryos). Embryos of 0.70 mm only reached maturity and germinated after 23 days culture, and the threshold embryo size for normal development was over 0.55 mm. Although such a discrepancy with published results could be attributed to variability in coconut milk composition, the results are supported by similarly poor viability and abnormal development seen by Chang (1963) in 0.50 mm embryos grown on White's medium and 10 per cent coconut milk.

Ziebur *et al.* (1950) observed that the addition of one per cent casein hydrolysate to Randolph and Cox's mineral medium, considerably increased embryo growth, but depressed proembryo development. The results here, have similarly shown that 0.05 to 0.30 per cent casein hydrolysate stimulates embryo development, and reduces the morphological threshold of viability. These effects were further enhanced by the inclusion of glutamine (400 mg l<sup>-1</sup>) in the medium. The importance of glutamine particularly, in the growth of the smallest proembryos, is endorsed by the results of Norstog (1961).

Although some of the modifications to Norstog's Medium I could induce superficially-normal embryo development, their morphology during later germination revealed that only those embryos grown on Norstog's Medium II approximated *in vivo* embryo growth. At a critical stage of development (0.25 to 0.30 mm) embryos attained viability on the latter medium, and after 10 days rapid growth, they reached mature proportions and germinated. The embryos continued to enlarge during the initial stages of germination, a process which is less likely to occur within the grain, owing to the physical restrictions of the husk. It is germane to note that in embryos of 0.25 to 0.30 mm in length, the primary region of the stem meristem is just established. All of the 0.20 mm embryos from the five *H. distichum* varieties tested failed to develop.

In attempting to explain the basis of the differences in embryo growth on these media, and the increasing sensitivity found in small embryos to the composition of the growth



medium, consideration will be given to the nature of the natural environment of the developing embryo. It should equally be said that in attempting to explain the results of *in vitro* embryo culture, we may deduce something of the nature and control of embryogenesis *in vivo*.

The use of sucrose as sole carbohydrate source in many embryo culture media (Narayanaswami and Norstog, 1964), is not easily explained, particularly when the sugar assimilation of many germinating embryos is taken into consideration. In germinating cereals glucose, released on hydrolysis of endosperm starch reserves, is actively accumulated by the embryo scutellum. It is subsequently converted to sucrose, and translocated from the scutellum to the apical meristems (Edelman, Shibko and Keys, 1959). If the main carbohydrate source of developing embryos were sucrose, then the scutellum would be required to show a flexibility in its transport mechanisms, to enable it to accommodate the changing assimilation pathways.

Limited analyses of the carbohydrate composition of barley grains has shown that the total reducing sugars reach a peak early in grain development, and then decline rapidly with the deposition of starch. Sucrose and fructans, in particular gluco-diffructose, comprise a major part of this fraction, while the concentration of fructose considerably exceeds that of glucose (Baxter and Duffus, 1973; Cerning and Guilbot, 1973).

The ability of very young embryos to utilize sucrose is supported by the early appearance of enzymes involved in sucrose cleavage, namely UDP- closely followed by ADP-sucrose synthetase, and the much later appearance of sucrose synthesizing enzymes (Duffus and Rosie, 1975).

The importance of sucrose in the culture medium must also be considered in the context of the control of osmotic potential. It has been possible to sustain the growth of immature cotton embryos by regulating the growth medium osmotic potential at -15 bars (1500 kPa), a value very similar to that of 12-day ovules, using either sucrose, sodium chloride or mannitol (Mauney, 1961). Germination could subsequently be induced by transferring the embryos to a sugar or salt-free medium, which may correlate with the natural rise in osmotic potential of the maturing embryo. A gradual reduction of sucrose concentration in both coconut milk and casein hydrolysate supplemented media, was successfully used in the present investigations to reduce the occurrence of precocious germination.

Amino acid metabolism in germinating embryos is well developed, which is essential if they are to depend on the limited range of amino acids provided by the endosperm reserve proteins, as a main source of reduced nitrogen. The biosynthetic autonomy of the developing embryo is undocumented, but as with the protein-synthesizing apparatus of the endosperm, it must depend on the amide and amino nitrogen translocated to the ear. The composition of the translocation stream will inevitably be influenced by the plant growth environment (Folkes, 1970), however analyses have been made of the free amino acid 'pool' in the endosperm of developing 'Bomi' barley (Brandt, 1975). The major amino acids present 13 days after fertilization, given in order of decreasing concentration were alanine, glutamine, glutamate, serine, threonine, glycine and proline.

The only growth medium to support the normal development and germination of >0.25 mm embryos was Norstog's Medium II. From the composition of the growth media in Table I it can be seen that the major nitrogen source in this medium is ammonium ions, which are supplied in the form of ammonium malate (pH 4.9). Low concentrations ( $\mu\text{M}$ ) of a few amino acids are also included in the medium, in addition to 2.7  $\mu\text{M}$  glutamine. The ability of the embryos to grow on this medium implies that the pathways for the transamination of Krebs' cycle intermediates and the further synthesis of other amino acids, are fully developed at a very early stage of development.

The failure of embryos to develop on Kasha's medium, where the major nitrogen sources are nitrate and ammonium ions may well stem from the form in which the ammonia is



supplied. In the analysis of the amino acid metabolism of soya-bean cell cultures, Gamborg (1970) noted that while low ammonium concentrations (2 mM) stimulated cell growth, high levels (20 mM) were inhibitory. It was suggested that, by inhibiting the Krebs' cycle, ammonia could reduce the flow of carbon skeletons and the supply of reduced pyridine nucleotides, both essential for amino acid synthesis. This theory was partly supported by the ability of citrate, when included in the growth medium, to overcome the inhibition. In Kasha's medium, unlike Norstog's Medium II, the ammonium ions are not balanced by a supply of Krebs' cycle intermediates.

The ammonium malate concentration of Norstog Medium II as compared with I is increased by 10-fold, and calcium nitrate is excluded. A decrease in the alanine concentration accounts for a halving in the amino acid concentration. Apart from alanine and glutamine the basis for the amino acid composition of these media is unclear since it bears little relationship to the predominant free amino acids in the developing grain.

If ammonium ions were the preferred form of reduced nitrogen, this might contribute to the poor growth of small embryos on Norstog's Medium I. Increasing the total amino nitrogen concentration three-fold by the addition of 0.3 per cent casein hydrolysate, considerably enhanced embryo growth, particularly in older embryos. It thus appears that the requirement for ammonia can partly be replaced by amino nitrogen. A combination of  $2.7 \mu\text{M}$  glutamine and 0.1–0.3 per cent casein hydrolysate, not only increased embryo growth, but also lowered the embryo size at the threshold of viability. These observations might be explained, when the preponderance of glutamine in the embryo environment, and the central role played by glutamine in linking carbohydrate and nitrogen metabolism, is borne in mind.

Coconut milk is included in growth media, for the induction and maintenance of callus from a wide variety of plant tissues, including *Lolium* and *Hordeum* pollen (Clapham, 1971) and *Daucus carota* (Wiggans, 1954); and for embryo culture. Owing to the undefined composition of the milk it is difficult to identify the basis of the plant growth effects. The cytokinins in coconut milk, of which 9- $\beta$ -D-ribofuransylzeatin is a major component (Letham, 1968) may well play an important part in callus induction and thus explain the frequency of abnormal development culminating in callus formation in the coconut milk supplemented media. Other potentially important constituents of coconut milk include myo-inositols, a prominent component of many endosperm tissues (Pollard, Shantz and Steward, 1961) and an amino acid fraction of which glutamate forms a large proportion (Pradera, Fernandez and Calderin, 1942). Any of these components of coconut milk may contribute to the enhancement of embryo growth.

From the experimental observations in this laboratory, the precise composition of the mineral medium is far less critical in determining the success of embryo culture, than the main nitrogen and carbohydrate sources. When Norstog's Medium I was replaced by White's (1943) mineral medium for the basis of the coconut milk and casein hydrolysate supplemented media, very similar embryo growth results were obtained. However, Norstog (1967) has shown that significant increases in embryo viability could be obtained when the concentration of either potassium chloride or potassium nitrate was raised 5-fold.

#### ACKNOWLEDGEMENTS

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## THE DEVELOPMENT OF ENDOSPERM AMYLOPLASTS DURING GRAIN MATURATION IN BARLEY

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**The ultrastructural changes accompanying endosperm maturation in barley are described with particular reference to the developing amyloplast. Starch synthesis within the young amyloplast was initiated at a number of sites which later coalesced into a single granule. By 14 days after anthesis two populations of amyloplast, large and small, were seen. These were present throughout endosperm maturation. From results using UDP-(U<sup>14</sup>C)—glucose introduced to the endosperm from the flag leaf during ear photosynthesis, it is suggested that the small amyloplasts do not arise from the large amyloplasts but that they constitute a separate population.**

Key words: *barley, endosperm.*

### INTRODUCTION

Amyloplasts from developing barley endosperm, in common with those from wheat<sup>1</sup> and from potato,<sup>6</sup> can be divided into two populations—large amyloplasts and small amyloplasts. Those from barley endosperm differ both chemically and in their associated enzymic activities.<sup>15</sup> It is therefore possible that the two populations have different origins. There is, however, considerable controversy regarding the initial stages of amyloplast development. Buttrose<sup>4</sup> has stated that in each plastid there occurs the initiation of one starch granule which then grows rapidly in size. This is followed later by the initiation of small amyloplasts in the stroma space. Earlier, May and Buttrose<sup>5</sup> had reported that the large amyloplasts of developing endosperm are initiated up to two weeks after fertilization, but not later, and the small only after 14 days. Unfortunately, no evidence was presented to support this. On the other hand, Badenhuizen<sup>1</sup> has suggested that amyloplasts arise from mitochondria. It has also been proposed<sup>3</sup> that the small amyloplasts are budded off from the large.

The presence of a membrane surrounding both the large and small amyloplasts and whether it remains until maturity is also disputed. Badenhuizen<sup>1</sup> suggested that the plastid membrane did not remain around the growing starch granule and later<sup>2</sup> proposed, using electron micrographic evidence, that the amyloplast appears to be a vesicle surrounded by a double membrane. Buttrose<sup>3</sup> showed electron micrographs of proplastids and amyloplasts at 14 days after anthesis showing a double membrane at both these stages. Seckinger and Wolf<sup>12</sup> found some protein attached to wheat starch granules, although not a continuous membrane, and Simmonds<sup>13</sup> also found some evidence for a membrane in the protein matrix from wheat endosperm.

Buttrose<sup>3</sup> in his electron microscope study of developing barley endosperm did not work with tissue older than three weeks after anthesis (in conditions where anthesis to maturity took 30 days) because of the difficulty of obtaining good sections after this age due to the drying out of the tissue.

The present work describes some of the ultrastructural changes taking place in barley endosperm throughout the developmental period to maturity. The electron micrographs of older tissue were, however, of relatively poor quality. In order to obtain further information on the initial stages of amyloplast development the differential incorporation of UDP-(U<sup>14</sup>C)—glucose into the separate populations of large and small amyloplasts was followed. These results were then correlated with the observed ultrastructural changes.

### MATERIALS AND METHODS

**Plant material.**—The barley used was *Hordeum distichum* (L) Lam. var. Julia. Ears were either used immediately or stored at  $-19^{\circ}\text{C}$  for a maximum of three months.

**Electron microscopy.**—Endosperms were dissected out of grain at various ages and cut into approximately 1 mm cubes. The prepared tissue was then fixed in 4% glutaraldehyde for 2 h, followed by staining in 2% osmium tetroxide for 2 h or fixed and stained in unbuffered 5% potassium permanganate for 2 min.<sup>10</sup> Dehydration was carried out in a series of aqueous ethanol solutions, the 70% alcohol containing 1% uranyl acetate to increase staining. The tissue was then embedded in TAAB embedding resin (TAAB Laboratories, Reading, Berks.). Sections were cut on an LKB Ultramicrotome III using a glass knife. Sections (500 Å) were cut from tissue which did not contain starch. Sections 600 Å–700 Å in thickness were cut when the tissue contained starch. The sections were collected on copper grids coated with necloidine and post-stained in Reynolds' lead citrate<sup>11</sup> for 20 min. After washing and drying, the sections were lightly shadowed with carbon. The microscope used was a GEC/AEI EM802 electron microscope.

**Uptake of carbon-14 by large and small amyloplasts.**—Carbon-14, in the form of UDP-(U<sup>14</sup>C)—glucose, was supplied to growing barley plants from a vial attached to the flag leaf. A slit was cut parallel to the veins in the flag leaf to allow the leaf to take up the solution more easily. The vial contained 0.25  $\mu\text{Ci}$  UDP-(U<sup>14</sup>C)—glucose in UDPG. The vial was left in place for 24 h, after which it was removed, and the plant allowed to develop until 25 days after anthesis (where the vial was attached before this age) or 50 days after anthesis when older plants had been used. Five endosperms were then removed from the treated ear and homogenised in 1.0 ml 0.15 M phosphate buffer, pH 7.3. The large and small amyloplasts were then separated on a sucrose gradient as described previously.<sup>15</sup> The amyloplasts were washed three times in distilled water and then suspended in 1.0 ml distilled water. 0.5 ml of this suspension was added to 5 ml liquid scintillant (NE 220, Nuclear Enterprises Ltd., Edinburgh) and counted in a Beckman Liquid Scintillation Counter at a counting efficiency of 73%.

### RESULTS

**Electron microscopy.**—Some of the ultrastructural changes accompanying amyloplast development in barley endosperm are shown in Figs. 1–6. With the exception of Fig. 6, these are typical of the results obtained on many occasions. At two days after anthesis amyloplasts could be seen in the endosperm, each containing many small starch granules (Fig. 1). Single starch granules surrounded by a membrane are also seen (Fig. 2). Cell walls, mitochondria and various osmophilic droplets are also seen at this stage. By three days after anthesis some of the starch granules were 0.5  $\mu\text{m}$  to 1.0  $\mu\text{m}$  and at six days a range of starch granule sizes was seen, varying from 0.25  $\mu\text{m}$  to 1.5  $\mu\text{m}$  along the longest axis. By 14 days after anthesis the two distinct size classes were first seen, the larger being about 11  $\mu\text{m}$  across and the smaller (Fig. 3) 3 or 4  $\mu\text{m}$  across. By 18 days many of the starch granules have coalesced and had begun



to fill the amyloplast (Fig. 4). The membrane in this case was very close to the starch granule. However, even at later ages (Figs. 5 and 6) the starch granule within the amyloplast may still be surrounded by a double membrane often with a considerable gap between the two. A further feature of endosperm

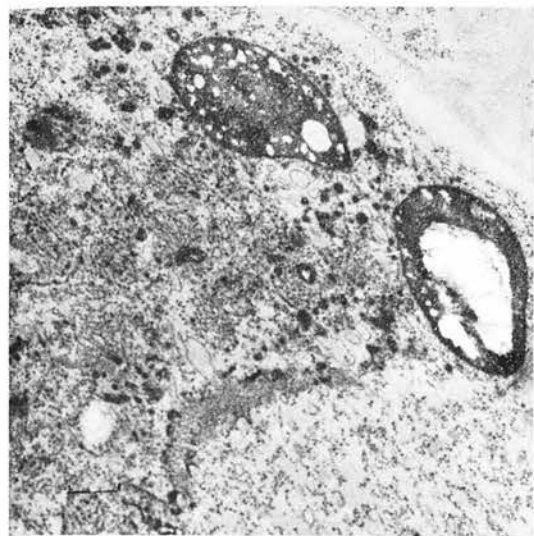


Fig. 1. Amyloplasts from 2 day endosperm showing multiple initiation sites of starch synthesis. Glutaraldehyde/osmium tetroxide fixation. Magn. 20,000 (Bar marker = 2  $\mu$ m)

development is that mitochondria appear to surround the immature amyloplast (Fig. 3) and that these persist, apparently undamaged, well into the maturation period (Fig. 5). Occasionally two starch granules may be seen within one double membrane. Amyloplasts with multiple starch granules are common in the early stages of maturation (Fig. 1) but at later stages, *i.e.* 38 days, they are a rarity (Fig. 6).

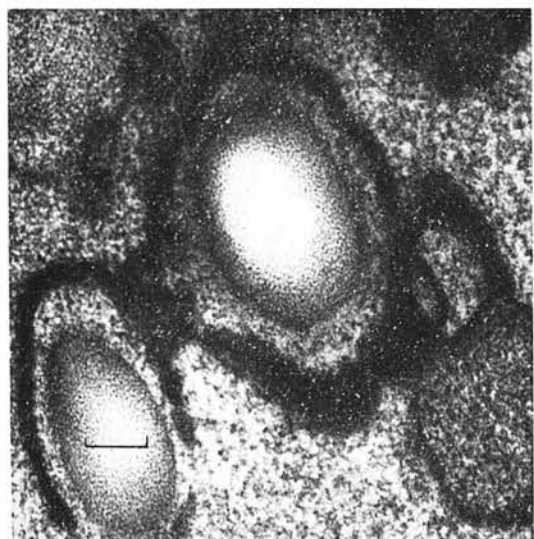


Fig. 2. Amyloplasts with well defined starch granules from endosperm 2 days after anthesis. Potassium permanganate fixation. Magn.  $\times 125,000$  (Bar marker = 0.05  $\mu$ m).

**Uptake of carbon-14 by large and small amyloplasts.**—When the original endosperm homogenate was counted before separation of the amyloplasts on a gradient, it was found that between 0.2% and 0.9% of the counts were taken up from the vial to the endosperm. The exact amount of UDPG taken up in this way varied slightly between experiments. Attaching the vial to the plants and cutting the flag leaf did not affect the normal development of the plant.

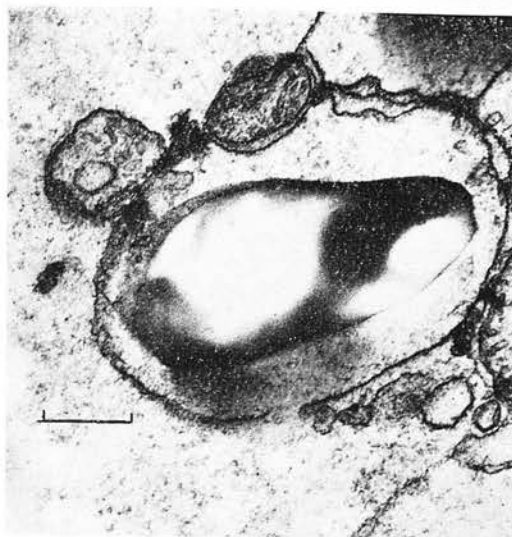


Fig. 3. Mitochondria lying close to the amyloplast membrane in endosperm 14 days after anthesis. Potassium permanganate fixation. Magn.  $\times 35,000$  (Bar marker = 2  $\mu$ m).

The results are shown in Table 1. The large amyloplasts were found to be labelled at 25 days after anthesis when the labelling had been done at all the different ages tried. When labelling was done at 35 days after anthesis the large amyloplasts were labelled by the time the gradient was done at 50 days after anthesis. The small amyloplasts were not found to be labelled at 25 days when labelling was done at earling or at 10 days after anthesis. When labelling was done at 14 and 24 days after anthesis there was a small, but significant, labelling of small amyloplasts by the time of separation at 25 days after

TABLE I. Radioactive labelling of large and small amyloplasts separated from immature endosperm after uptake of  $^{14}\text{C}$  by the flag leaf

Age at which labelling was carried out	Amyloplast fraction	c.p.m./ml of amyloplast suspension (average of at least 2 determinations)
Earing*	large	244
	small	0
10 days after anthesis*	large	198
	small	4
14 days after anthesis*	large	586
	small	30
24 days after anthesis*	large	206
	small	33
35 days after anthesis**	large	73
	small	0

\* Large and small amyloplasts separated and counted at 25 days after anthesis.

\*\* Large and small amyloplasts separated and counted at 50 days after anthesis.

anthesis. There was no labelling in the small amyloplasts when labelling was carried out at 35 days after anthesis. At earling no amyloplasts could be seen under the light microscope. At 10 days all the amyloplasts seen were small amyloplasts. The two populations of amyloplasts were not seen under the light microscope until 14 days after anthesis.

#### DISCUSSION

The electron micrographs obtained here show the presence of an amyloplast double membrane both at early and late stages in development. Although at early stages cytoplasm

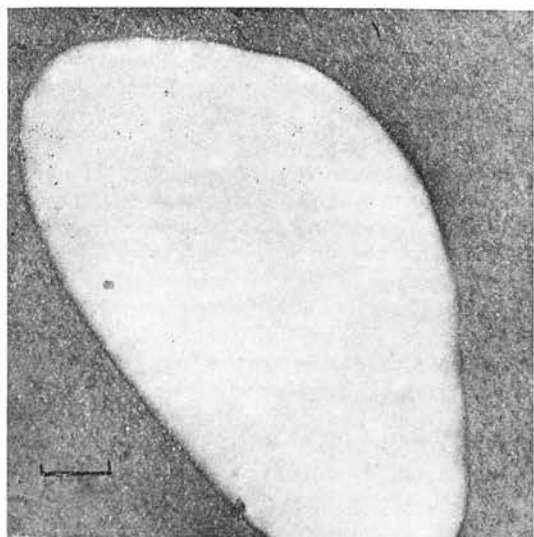


Fig. 4. Amyloplast with close lying membrane 18 days after anthesis. Potassium permanganate fixation. Magn.  $\times 31,000$  (Bar marker =  $2 \mu\text{m}$ ).

may be seen between the membrane and the starch granule, later in development the membrane generally lies very close to the granule. In some cases the membrane is not complete, perhaps because of damage caused during cold storage. The presence of protein and lipid in isolated washed amyloplast fractions was additional (not shown) evidence for the presence of a membrane even at later stages of development.

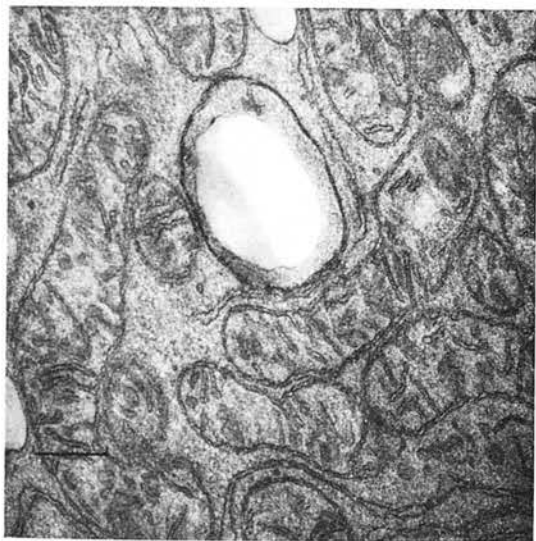


Fig. 5. Endosperm 38 days after anthesis showing the presence of many mitochondria, some lying close to the amyloplast double membrane. Potassium permanganate fixation. Magn.  $\times 35,000$  (Bar marker =  $2 \mu\text{m}$ ).

In amyloplasts at two and three days after anthesis many small granules can be seen. This suggests that in the amyloplast of immature barley endosperm at least, starch material is initiated at a number of sites which then coalesce to a single granule. The presence of two granules within one membrane, although possibly an artefact of preparation, and of the extra membrane in Fig. 6 may indicate that the plastid is dividing. The phenomenon of Fig. 6 was observed, however, on only one occasion. A rudimentary lamellar structure could occasionally be seen within the amyloplast (see for example Fig. 5). This, together with the double membrane, demonstrates its similarity to the proplastid from which both amyloplasts and chloroplasts may be derived. It may also be that, under certain conditions, the amyloplast has the potential to become a chloroplast.

While it was confirmed that the two populations of amyloplast were seen at, and after, 14 days after anthesis, there was no indication from the present work that the small amyloplasts develop directly from the large, for example by budding,<sup>3</sup> or, indeed, that they are derived from mitochondria.

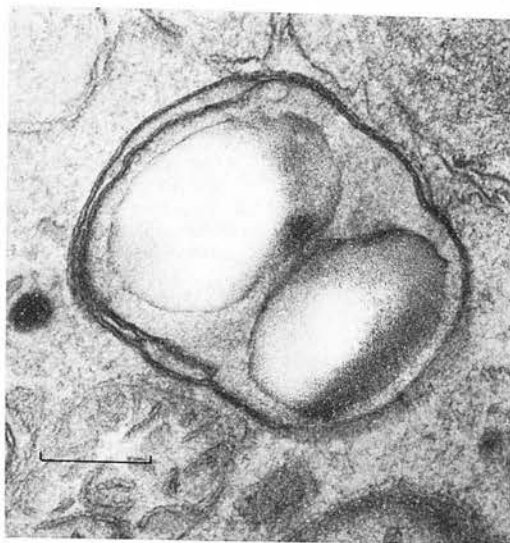


Fig. 6. Amyloplast containing two starch granules with well defined double membrane(s). Potassium permanganate fixation. Magn.  $\times 50,000$  (Bar marker =  $2 \mu\text{m}$ ).

The presence of mitochondria is not surprising since respiratory processes are known<sup>7</sup> to be active during the major part of endosperm maturation. Their close association with the amyloplast indicates that the respiratory substrate may be degraded amyloplast starch rather than sucrose or soluble oligosaccharides. A potentially very active starch hydrolysing system is present<sup>5</sup> in all parts of the immature grain during maturation.

It is possible to devise at least three theories for the origin of the two types of amyloplast in immature endosperm. One is that A-type amyloplasts—i.e. those which later become large—are produced throughout the growth of the barley grain and grow until the barley reaches maturity. The small ones are then those which are produced later in development.

A second theory is that both 'A' and 'B' types of amyloplasts are produced at all stages of development of the barley. The 'B' type would stay small, whereas the 'A' type would grow larger throughout development. These two theories, however, do not easily account for the fact that two types of amyloplast are first seen at around 14 days after anthesis.

The third theory is that one type (the 'A' type) is produced throughout growth but that the 'B' type of small amyloplasts is produced at around 14 days after anthesis and thereafter stay small. It was suggested by May and Buttrose<sup>8</sup> that this second small type may be produced simply because of lack of space in the endosperm at this age for larger amyloplasts to be produced.

Different results can be predicted using each of these three theories if the amyloplasts are labelled with carbon-14 at various stages during development.

According to the first theory the large amyloplasts should be labelled at all stages and the small amyloplasts labelled only when labelling was done at 24 and 35 days after anthesis.

According to the second theory both large and small amyloplasts would be labelled, regardless of the age at which labelling was carried out. By the third theory the large amyloplasts should again be labelled at all stages but with the small amyloplasts labelled at 14, 25 and possibly 35 days. The results indicate that of these three theories the third is most likely, i.e. the amyloplasts which were labelled at the earing

stage and 10 days after anthesis grew to become large amyloplasts, the small being produced later. Of the amyloplasts labelled at 14 and 24 days, some grew to become larger amyloplasts while others remained small. The fact that the small amyloplasts were not labelled when labelling was done at 35 days after anthesis indicates that all the small amyloplasts of both types have been produced by this age. Thus the first type, type A, are young large amyloplasts and the type B amyloplasts constitute a separate population.

The method used (that of attaching a vial of solution to the flag leaf) was found to be the most suitable for this experiment. Injecting the grain was unsuccessful, since it was impossible to inject an exact amount of liquid into the grain at successive attempts. Sodek and Wilson<sup>14</sup> rejected results obtained when <sup>14</sup>C-labelled amino acids were injected into corn endosperm suggesting that the conflicting results which they obtained might be partly accounted for by the different physical and metabolic states of the endosperm at the various stages in development.

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# Metabolism of Ammonium Ion and Glutamate in Relation to Nitrogen Supply and Utilization during Grain Development in Barley<sup>1</sup>

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## ABSTRACT

Changes in the activity of a number of enzymes concerned with amino acid synthesis and metabolism were recorded for the endosperm, testa pericarp, and embryo of developing barley (*Hordeum distichum* L.) grains. Both glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase activities were present in all tissues and at all ages examined. Glutamate dehydrogenase activity was largely confined to endosperm while glutamine synthetase activity was mainly in the testa pericarp.

Ammonium ion concentration was maximal in endosperm by 20 days after anthesis. Glutamate concentration varied in endosperm and was in the range of 3.5 to 8.5 mM between 20 and 45 days after anthesis. Significant levels of ammonium ion and glutamate were also present in the testa pericarp over the major part of the developmental period.

Cereal grain maturation is accompanied by a net synthesis and deposition of macromolecules such as starch and protein. The metabolic events associated with starch biosynthesis have been subject to much investigation in a number of cereals including maize (31), rice (25), and barley (2). In the case of protein, the possibility of improving protein quality by selection of mutant varieties has stimulated interest in the synthesis of cereal proteins, notably in wheat (13) and maize (16). Little is known, however, of the factors regulating the supply of nitrogen to the grain, and the pathways involved in its incorporation into grain protein. Furthermore, the source and form of nitrogen supplied to the developing grain are not clear. It has been suggested (29) that because storage organs transpire relatively slowly, the bulk of inorganic ions they receive comes via the phloem rather than the xylem. That this may be the case in barley is borne out by the results of Archbold (1), who showed that the dry matter entering the developing grain is derived mainly from photosynthesis after grain emergence. The nitrogen required for development in lupin seeds moves in the phloem (23), the major compounds present being similar to those found in maize xylem sap (15). Glutamate/glutamine and aspartate/asparagine were the predominant amino acids. In phloem, as in xylem (15), the relative amounts present of the various nutrients will no doubt depend on the physiological status and inorganic nutrition of the plant.

The mode of incorporation of nitrogen into the amino acids of higher plants is also a matter of some controversy. Mifflin (20) have suggested that the pathway of preference is via glutamine synthetase rather than via glutamate dehydrogenase. However, there is no unequivocal evidence regarding the mechanism of nitrogen

assimilation in different plants or different tissues. For example, it has been pointed out (20) that at the levels which might be required for appreciable glutamate dehydrogenase activity, ammonium ion would be present in toxic amounts. However, even if glutamate dehydrogenase acts *in vivo* at around half-maximum velocity, thus requiring relatively high ammonium concentrations, the substrate and enzyme may well be restricted to a different cell compartment with minimum effects of ammonia on other processes.

The work described in this paper represents a further investigation of the roles played by glutamate and ammonium ion in relation to nitrogen supply and utilization during barley grain development. The observed changes are then correlated with known changes in metabolism taking place in barley grains developing under the same conditions.

## MATERIALS AND METHODS

**Plant Material.** The two-row barley *Hordeum distichum* L. cv. Julia was used throughout. Plants were grown in the greenhouse under mercury vapor lamps with an 18-hr illumination period. Under these conditions the period between anthesis and maturity took 60 days. The method used to determine the date of anthesis was that of Merritt and Walker (19). Immature grains were used either fresh from the ear or after storage at -18°C for periods of not more than 3 months.

**Extraction of Enzymes.** A minimum number of 10 endosperms, embryos, or testa pericarps was used for each assay. Endosperms (including the aleurone layer) and embryos (including the scutellum) were dissected out by hand after removal of the grains from intact ears. As soon as possible after dissection the tissues were suspended in a minimum volume of the appropriate buffer at 4°C and homogenized completely using a Potter-type, hand-held, all glass homogenizer. Testa pericarps were similarly homogenized in a Griffiths' ground glass hand-held homogenizer. The buffer to be used in the subsequent enzyme assay system was used as the extraction medium in each case. Homogenization was continued until tissue disruption was complete and soluble protein extraction maximal. Suspensions were centrifuged immediately at the speeds indicated in the assay methods or at 10,000g for 3 min on a Beckman microfuge. Since the object of this study was to measure enzyme activity as nearly as possible under *in vivo* conditions, the tissues were diluted as little as possible and no stabilizing factors were added to the homogenizing medium.

**Enzyme Assays.** Glutamate-oxaloacetate transaminase (4) and glutamate-pyruvate transaminase (5) were assayed by standard methods. L-Glutamate dehydrogenase was assayed as described by Strecker (28) except that 0.1 M glycylglycine buffer was used. Glutamine synthetase was assayed by the method of Rowe *et al.* (26), except that the reaction mixture (1 ml) contained 50 μmol of HEPES buffer (pH 7.2). Controls in which enzyme, ATP, and

<sup>1</sup> This work was supported by the Agricultural Research Council.



glutamate were separately omitted were carried out. Glutamate synthetase was assayed using the reaction conditions of Lea and Milfin (17) except that the reaction mixture (1.6 ml) contained 80  $\mu$ mol of HEPES buffer (pH 7.5) and 0.34  $\mu$ mol of NADH. NADH oxidation was measured by the decrease in  $A$  at 340 nm. Nitrate (14) and nitrite (18) reductases were assayed by standard methods except that the reaction mixture for nitrite reductase contained 7.5 mM HEPES buffer (pH 7.8). Crude proteolytic activity was measured in buffered (pH 7.5) extracts of endosperm as described by Penner and Ashton (24). Enzyme activity was proportional to the amount of extract used. The mean standard deviation of all results was around 15%.

**Assay of Ammonium Ion and Glutamate.** Ammonium ion was assayed enzymically using glutamate dehydrogenase (30). Determinations were carried out as quickly as possible. The total time between dissection of endosperms and completion of experiments, including samples and controls, was around 40 min. The time between homogenization of tissue and initial spectrophotometric measurement was around 10 min. Sensitivity of the method using standard  $\text{NH}_4\text{Cl}$  solution was found to be greatest at pH 8. The assay was calibrated using different concentrations of  $\text{NH}_4\text{Cl}$ . The reaction rate was proportional to ammonium ion concentration in the range of 0.02 to 0.1  $\mu$ mol/ml. Tissue homogenates were tested for deaminase activity using glutamine as substrate at pH 8.

Glutamate was measured enzymically, using glutamate dehydrogenase, in endosperm and testa pericarp after initial tissue extraction with 0.5 N  $\text{HClO}_4$  as described by Bernt and Bergmeyer (6).

The concentration of glutamate and ammonium ion in endosperm was calculated, using the figures for water content of intact endosperms obtained by drying a known weight of fresh endosperms at 80 C to constant weight.

## RESULTS

The activity of the enzymes glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase in endosperm, testa pericarp, and embryo is shown in Figures 1 and 2. Measurable activity was recorded in all tissues and at all ages studied. In both cases activity was considerably greater in the endosperm than in the other two tissues. Results are expressed as total activity/tissue part and not on a protein or dry wt basis since these are variable quantities. For the first 30 days of embryo development glutamate-pyruvate transaminase activity was relatively low but rose rapidly thereafter to a steady maximum by 45 days. Testa pericarp activity fell to relatively low values by 50 days and endosperm activity fell from its maximum value of 3.8  $\mu$ mol/min·endosperm at 40 days after anthesis to 0.1  $\mu$ mol/min·endosperm at 60 days after anthesis. The pattern of activity for glutamate-oxaloacetate transaminase was similar to that for glutamate-pyruvate transaminase. Activity was present in all tissues and at all ages studied.

The major part of grain glutamate dehydrogenase activity was present in the endosperm (Fig. 3) with a maximum value at 40 days after anthesis. Activity in the testa pericarp was observed only during the first 30 days of development. Embryo activity increased slowly until 50 days after anthesis, thereafter remaining constant.

In sharp contrast to these results, only a small part of total grain glutamine synthetase was found in the endosperm (Fig. 4). Measurable activity was recorded only at 12 and 25 days after anthesis. Over 95% of activity was present in the testa pericarp during the greater part of grain development, the maximum value being attained at around 30 days after anthesis. Addition of endosperm extract to the testa pericarp enzyme did not inhibit glutamine synthetase activity. Embryo activity was very low compared to that in the testa pericarp over most of the development period. A peak of activity at 35 days and by 45 days activity was similar to that found in the testa pericarp.

Ammonium ion contents of endosperm and testa pericarp were

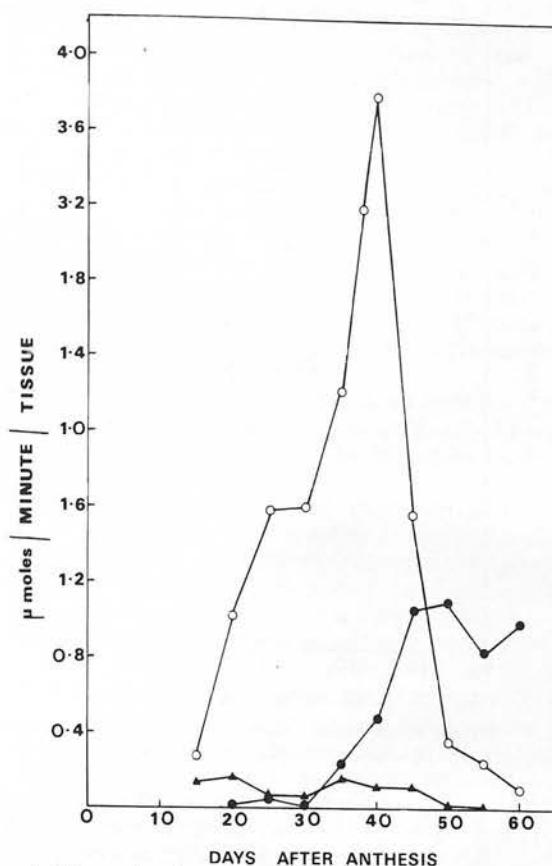


FIG. 1. Changes in glutamate-pyruvate transaminase activity in endosperm (○), testa pericarp (▲), and embryo (●) during grain development.

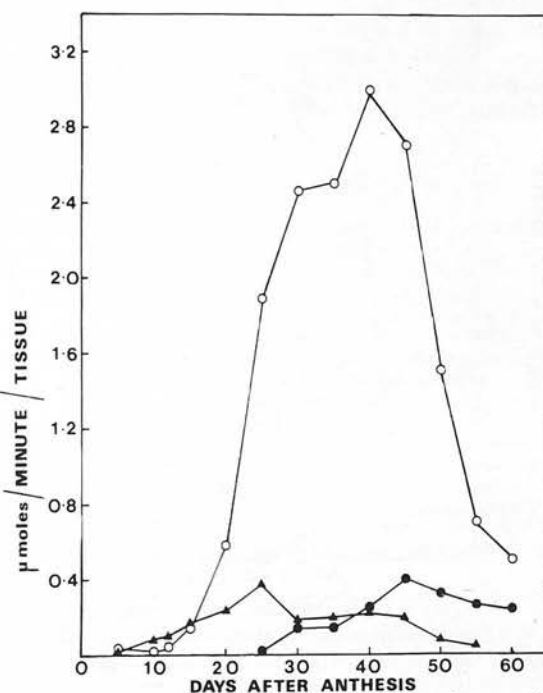


FIG. 2. Changes in glutamate-oxaloacetate transaminase activity in endosperm (○), testa pericarp (▲), and embryo (●) during grain development.

very similar until 45 days after anthesis when levels in the endosperm fell dramatically (Fig. 5). By 50 days after anthesis, no ammonium ion could be detected in either tissue. Ammonium ion concentration in the endosperm increased rapidly from very low

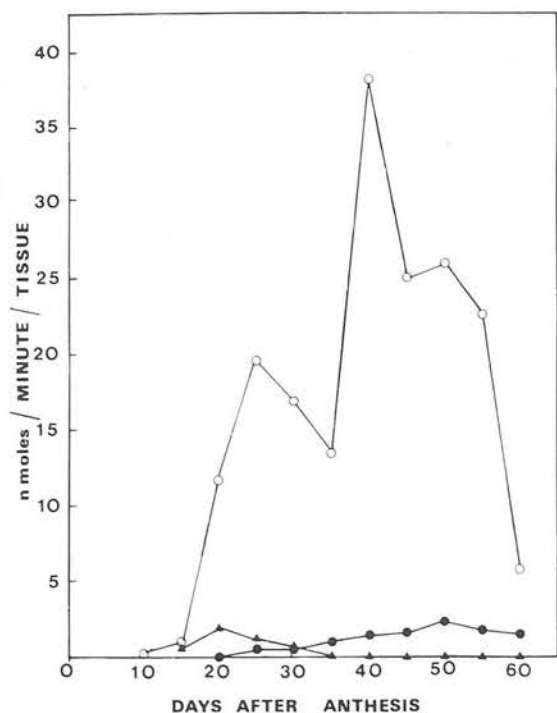


FIG. 3. Changes in glutamate dehydrogenase activity in endosperm (○), testa pericarp (▲), and embryo (●) during grain development.

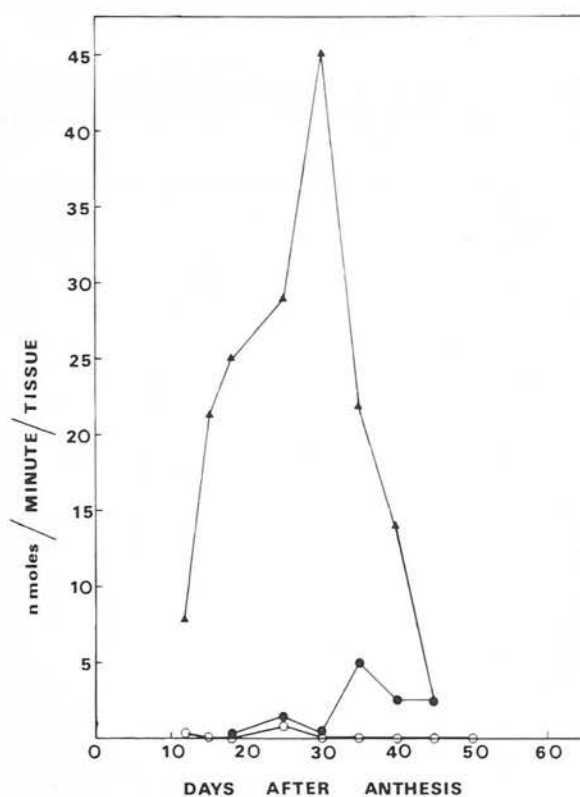


FIG. 4. Changes in glutamine synthetase activity in endosperm (○), testa pericarp (▲), and embryo (●) during grain development.

levels at 15 days to a maximum value of 40 mM at 20 days after anthesis, falling off thereafter to almost zero by 35 days after anthesis. Due to the high water content of the testa pericarp, at least during the first 30 days of development, and its high rates of water loss following separation from the grain, it was not possible to obtain accurate values for ammonium ion concentration in this

tissue. Deaminase activity, as measured by release of ammonium ion after incubation of endosperm extracts with glutamine, was not detected.

Between 7 and 15 days after anthesis glutamate was present solely in the testa pericarp; but by 20 days after anthesis the greater part was present in the endosperm, levels falling off sharply by 50 days after anthesis (Fig. 6). Small but detectable levels of glutamate were present in embryos throughout development. Glutamate concentration in the endosperm was maximal at 25 days after anthesis but did not decrease very greatly until 45 days after anthesis. Pericarp glutamate concentration is not shown, for the reasons described above. Significant levels of glutamate synthetase were not detected in endosperm or testa pericarp by the methods used. Neither nitrate nor nitrite reductase activity was detectable in testa pericarps at 25 days nor in endosperms at a number of stages after anthesis. Both enzymes were present in high activity in identical extracts of flag leaf.

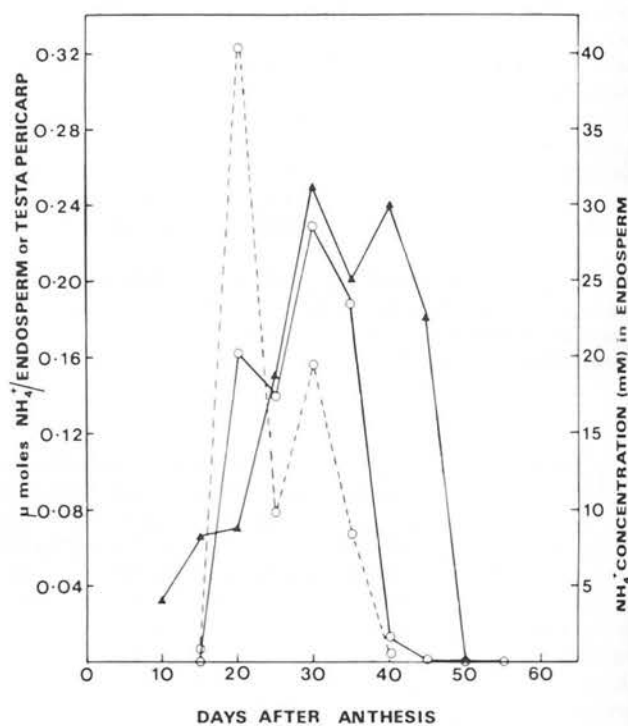


FIG. 5. Changes in ammonium ion amount (○), and concentration (○--○) in endosperm and ammonium ion amount (▲) in testa pericarp during grain development.

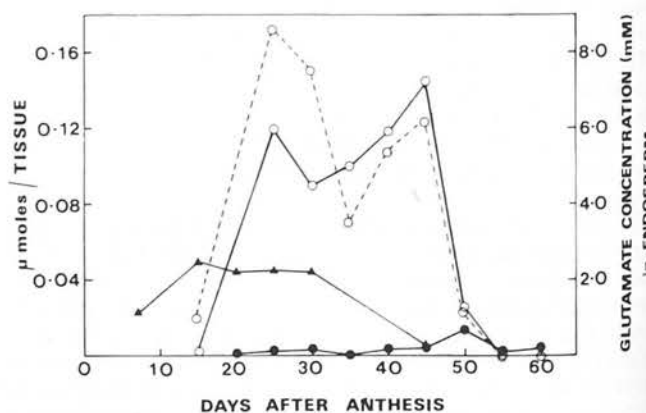


FIG. 6. Changes in glutamate amount (○), and concentration (○--○) in endosperm and glutamate amount in testa pericarp (▲) and embryo (●) during grain development.

## DISCUSSION

The early stages of grain development are associated with a wide range of morphological and biochemical changes. After the initial period of free nuclear division within the embryo sac (1-2 days) cell wall formation commences and is soon followed by the appearance of amyloplasts, protein bodies, and mitochondria (8). In the later stages of maturation the cells become filled with amyloplasts and any nuclei present become obscured. It is not surprising, therefore, that biochemical processes associated with nitrogen metabolism are active during the initial stages of differentiation. The early appearance of enzymes of amino acid metabolism, such as the transaminases, suggests that the nitrogen supplied to the grain may not be in the form of a complete range of amino acids in the correct proportions but rather in a form which requires much metabolic transformation before incorporation into grain protein. The apparent absence of nitrate and nitrite reductase activity from endosperm and testa pericarp suggests that nitrate may not be involved directly in grain nitrogen metabolism. Since both enzymes were present in the flag leaf, inorganic nitrogen may be supplied to the grain as ammonium ion rather than as nitrate. This is in agreement with the work of Pate and Sharkey (23) who showed that, while present in xylem, nitrate was not detectable at any time in phloem which may be the major source of grain nutrients. Ammonium ion is certainly found in the endosperm during the early stages of grain development and could be utilized there by glutamate dehydrogenase. The equilibrium lies in favor of glutamate synthesis and the acceptor is  $\alpha$ -ketoglutarate which, since respiratory processes are active (12) in developing endosperm, would be readily available. It may be that the supply of 2-oxoglutarate is itself regulated by ammonium ion concentration since it has been reported (3) that glycolysis, and hence the supply of carbohydrate for Krebs' cycle intermediate synthesis, may be activated by ammonium ions. Certainly the marked increase and decrease in glycolytic enzyme activity observed (12) during endosperm development follow the increase and decrease in ammonium ion concentration of endosperm described here. Endosperm ammonium ion concentration falls almost to zero by 40 days after anthesis and is closely followed by glutamate concentration, yet endosperm nitrogen is still accumulating (10). It may be that the relatively high glutamate dehydrogenase activity, which is maintained almost to maturity, compensates for the low levels of substrate present.

In contrast to the endosperm, very little glutamate dehydrogenase activity was detected in the testa pericarp, and then, only in the first 30 days of maturation. However, glutamine synthetase activity, which was largely undetectable in endosperm, was present. Ammonium ion and glutamate, both substrates for glutamine synthetase, were also detectable in significant amounts in testa pericarp from 10 days after anthesis. It may be that this is an example of compartmentation, where an enzyme is confined to a tissue in which metabolic conditions, unlike those elsewhere, are optimal for its activity. Cytoplasmic conditions in the testa pericarp will be very different from those in the endosperm since active photosynthesis in the testa pericarp will be accompanied by extrusion of hydroxyl ions from the chloroplasts so that the pH will tend to be alkaline, about pH 8, the optimum (22) for Mg-dependent glutamine synthetase. In the endosperm, by contrast, the pH is acidic, about pH 6.5, and at which the Mg-dependent glutamine synthetase will be relatively inactive and probably unstable. Since Mg is present in both tissues in much greater amounts than Mn (10, 11), the Mn-dependent glutamine synthetase activity, which has a pH optimum of about 5 (22), may be ignored. The sharp drop in testa pericarp glutamine synthetase activity coincides with a similar sharp drop in Mg (10) and Mn (11) levels in that tissue. The reverse situation is observed during embryo development where a steadily increasing Mg level (10) is accompanied by increasing glutamine synthetase activity. Gluta-

mine synthetase may be activated by products of photosynthesis such as ATP and NADPH (27). The pericarp is a photosynthetic tissue while the endosperm, although synthesizing ATP and NADPH via the glycolytic and pentose-P pathways (12), may maintain lower intracellular levels of these substances than the testa pericarp, in view of their requirement by the pathways of carbohydrate, lipid, and protein synthesis. Again, glutamine synthetase activity will be favored in testa pericarp. Certainly glutamine synthetase activity, as well as glutamate levels in the testa pericarp, fell markedly from 30 days after anthesis, a stage coinciding with decrease in Chl content and photosynthetic activity (21). The localization of glutamine synthetase in the testa pericarp is in accordance with our knowledge of the metabolic properties of that tissue and of the endosperm.

That glutamine synthetase inactivity in the endosperm may be the result of the absence of activators rather than the presence of inhibitors is shown by the failure of an endosperm tissue extract to inhibit the testa pericarp enzyme.

At no time could significant NAD-dependent glutamate synthetase activity be detected in endosperm or testa pericarp. Mifflin (20), however, has reported (unpublished results) the presence of this enzyme in immature barley endosperm. Levels of activity were not presented. Since the enzyme may be difficult to assay, particularly in unpurified extracts such as were used here, our inability to demonstrate activity is perhaps not surprising. This enzyme is found in a wide range of microorganisms (7) and, if measured activities are low, evidence that activity is not due to bacterial or other contamination would need to be presented.

As maturity approaches, it is clear that the processes of amino acid synthesis and metabolism are increasing in the embryo. This is consistent with observed increases in nucleic acid (9) and nitrogen deposition (10) in this tissue. It is clear that the mechanism controlling nitrogen metabolism in embryos is distinct from that in the endosperm and testa pericarp where activities are switched off as early as 30 days after anthesis.

In conclusion, the separate tissues of the developing grain may have distinct but interdependent pathways of amino acid metabolism. The changes observed in nitrogen metabolism can be correlated closely with known changes in metal ion content, substrate concentration, carbohydrate metabolism, and photosynthesis during grain development.

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# Variation in Amyloplast Size, Relative Numbers and Carbohydrate Composition During Endosperm Development in Sweet Corn

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P. H. Jennings, Amherst

The fractionation of immature sweet corn endosperm amyloplasts into seven size classes throughout kernel development is described. The numbers in each size class, expressed as a percentage of the total, have been followed throughout development. In addition, the amylose content of the different size classes has been determined and in each case expressed as a percentage of their  $\alpha$ -glucan content. The increase observed in percent amylose content of total amyloplast  $\alpha$ -glucan during kernel maturation may be due both to changes in the relative numbers of the different size classes as well as to changes in their amylose content.

**Änderung der Amyloseplastengröße, der relativen Anzahl und der Kohlenhydratzusammensetzung während der Endospermentwicklung von Süßmais.** Die Fraktionierung der Endospermamyoplasten von unreifem Süßmais in sieben Größenklassen während der Kornentwicklung wird beschrieben. Zusätzlich wurde der Amylosegehalt der einzelnen Größenklassen bestimmt und jeweils als Prozentgehalt ihres  $\alpha$ -Glucangehaltes ausgedrückt. Die beobachtete Zunahme des Amylosegehaltes bezogen auf das gesamte Amyloplastglucan dürfte sowohl auf die Änderung der relativen Anzahl der verschiedenen Größenklassen als auch auf die Änderung ihres Amylosegehaltes zurückzuführen sein.

## 1 Introduction

The starch content of cereal grains increases steadily during their development, generally accounting for around 65 to 75% of grain dry weight at maturity. The amount of amylose relative to the amount of starch also increases throughout grain development in barley [1] wheat [2] and maize [3, 4]. The observed changes in amylose content may have a number of explanations. Firstly, the amylose percentage of all amyloplasts might change, where each has the same composition at all developmental stages. Secondly, where amyloplasts are of differing composition at the same stage of development, the change in percentage amylose content may be due to changes in their individual amylose contents or to changes in their relative numbers during development, or to a combination of both. The former may be the case in wheat, since at maturity there is no significant difference in percentage amylose content of the starch of the small and large granules [5]. During barley endosperm development, on the other hand, the two types of amyloplast have been reported [6] to change in starch composition, with the large initially having a higher

amylose content than the small. The amyloplasts of maize, unlike those of barley and wheat are present in a range of sizes. Analysis of these at maturity has shown that the amylose content decreases as granule size decreases [4, 7].

Thus, there is evidence to suggest that maize endosperm starch granules may differ in polysaccharide composition, shape, and biosynthetic properties. It is not known however, whether the sugary mutation is associated with an increase in amylose content during development and whether or not the granules change in composition or relative number throughout development. Additionally the sugary mutation is of particular interest since the endosperm accumulates phyto-glycogen partly in the globular amyloplasts [8] and partly in very small granules which sediment between 600 xg and 2,000 xg [7].

In the present work a method for the rapid and gentle fractionation of immature sweet corn amyloplast is described. The changes taking place in amyloplast size, composition, and relative number are discussed in relation to the observed changes in amylose content during endosperm maturation.

## 2 Materials and Methods

### Plant Material

Sweet corn (*Zea mays* L.) var. Seneca Chief from the Harris Seed Company was grown in the greenhouse under standard conditions. All plants were self pollinated with only one ear from each plant being fertilized. Ears were harvested at 5, 10, 15, 20, 25, 30, 35 and 40 days post pollination and used either fresh or after not more than one month at  $-20^{\circ}\text{C}$ .

### Fractionation of Amyloplasts

The outer layers of pericarp, together with the embryo, were first removed from the intact kernels. The resulting endosperms were then suspended in ice cold 10% (W/V) sucrose solution and homogenized manually in a Potter type all glass, ice-cooled homogenizer. After filtration through 6 layers of cheesecloth the filtrate was centrifuged at  $1,160 \times g$  for 5 min. The pellet consisted of amyloplasts of all sizes and will be referred to as the unfractionated amyloplast pellet. The number of endosperms used was varied from 15 in 3.0 ml of 10% sucrose at 15 days post-pollination to 10 in 4.0 ml at 40 days. The unfractionated amyloplast pellet was resuspended in 3.0 ml of 10% sucrose and 1.0 ml of this layered on top of discontinuous sucrose gradient consisting of 2.0 ml each of 70%, 60%, 40% and 20% sucrose in a 15 ml polypropylene centrifuge tube. This was centrifuged for 5 min at  $1,160 \times g$  in a swing-out head by accelerating rapidly and decelerating slowly in the absence of braking power. After centrifugation, amyloplasts could be seen as thin white layers concentrated at the interfaces, and additionally as a pellet at the bottom of the tube. The layer between 10% and 20% sucrose will be referred to as amyloplast size class 1. Similarly that between 20% and 40% was size class 2, between 40% and 60% size class 3 and between 60% and 70% size class 4. The layers were removed using a Pasteur pipette, the pellet resuspended in 2.0 ml of 10% sucrose, layered on a further gradient of 2.0 ml each of 90% and 80% sucrose and centrifuged as above. In this case the 80% sucrose contained 0.1-M NaCl which prevented aggregation and subsequent sedimentation of smaller amyloplasts into the 90% layer. The white layer between the 10% and 80% sucrose will be referred to as size class 5, that between 80% and 90% as size class 6 and the pellet as size class 7. After dilution with an equal volume of distilled water, and sedimentation of the amyloplasts by centrifugation, the pellets were washed at least twice by resuspending in ice-cold distilled water and centrifuging until soluble carbohydrates were removed. The final fractions were not a quantitative recovery of all amyloplasts of a single size but rather were representative of all amyloplasts of that size.

### Amyloplast Number

The method for counting amyloplasts was a modification of that described by May and Buttrose [9]. A known volume of amyloplasts was diluted with a known volume of iodine solution (0.2%  $\text{I}_2$  in 2% KI). After vigorous mixing a portion was taken up into a wide bore pipette which was held horizontally to prevent differential sedimentation. A drop was placed on a haemocytometer slide (depth 0.1 mm) and covered immediately with a cover slip. The number of amyloplasts within an area of  $40 \times 10^3 \mu\text{m}^2$  was determined for each sample using a Zeiss light photomicroscope at a magnification of 1250 diameters. Numbers of different sized amyloplasts were estimated in unfractionated amyloplast pellets and in layers of uniform size after removal from the gradients. Only particles staining with iodine were counted.

### Amyloplast Size

Amyloplasts were stained with iodine and examined under a magnification of 1250 diameters. Measurements of size were made using an ocular micrometer and checked by calibration of this against an improved Neubauer haemocytometer grid. Between 85 and 90% of amyloplasts in each size class were of similar size.

### $\alpha$ -Glucan and Amylose Determinations

Total  $\alpha$ -glucan was determined in amyloplast fractions, washed at least twice by ice-cold distilled water, using the anthrone-sulphuric agent reagent [10] and amylose, after alkaline extraction, by the blue value method of Gilbert and Spragg [11]. Amylose content was determined by reference to a standard curve using pure amylose. All results were averages of 3 separate experiments within which amylose and total  $\alpha$ -glucan determinations were duplicated.

## 3 Results

It was impossible to obtain sufficient amyloplasts for analyses until 15 days post-pollination. Thereafter, the amylose content of the unfractionated amyloplast pellet increased steadily (Table 1) though the differences between consecutive values were not statistically significant in most cases. The  $\alpha$ -glucan content of the whole amyloplast fraction is shown for comparison. This increased rapidly throughout development. Thus, although the relative amount of branched polysaccharide to amylose in amyloplast starch fell throughout development, the total amount increased.

Table 1.

Changes in Amylose Content of Unfractionated Amyloplasts during Endosperm Development in Sweet Corn.

Unfractionated amyloplasts (see Materials and Methods) isolated at different stages throughout development were washed 3 times with ice cold distilled water before analysis. Results are the mean  $\pm$  standard deviation of 3 separate experiments within which amylose and  $\alpha$ -glucan determinations were duplicated.

Days post-pollination	Amylose content <sup>1)</sup> of total amyloplast $\alpha$ -glucan (% w/w)	Total $\alpha$ -glucan content <sup>1)</sup> of amyloplasts (mg/endosperm)
15	10	$1.4 \pm 0.5$
20	13	$5.8 \pm 1.4$
25	16	$11.0 \pm 1.6$
30	18	$15.4 \pm 3.6$
35	23	$17.0 \pm 0.3$
40	21	$30.0 \pm 4.7$

1) Average standard deviation  $\pm 1.8$ .

Amyloplasts from the sucrose density gradient were found to increase in size with increasing sucrose density. Those in size class 1 from the top of the gradient were not greater than  $0.8 \mu\text{m}$  and those in size class 7 were  $10 \mu\text{m}$  or larger. Very few exceeded  $15 \mu\text{m}$ . Dimensions of all size classes are shown in Table 2. The percentage amylose content of amyloplasts at any one age increased steadily with increasing amyloplast size at all ages examined (Table 2). In addition, amyloplasts in size classes 1 to 5 showed an increase in amylose percentage during development, with a maximum at 25 days post-pollination. Values fell thereafter. The overall pattern was, however, statistically significant only in size class 2. In classes 2, 4, 5, and



Table 2.  
Amylose Content as % (W/W) of Total  $\alpha$ -glucan, in Amyloplasts of Different Size Classes during Endosperm Development in Sweet Corn. Results are the mean  $\pm$  standard deviation of 3 separate experiments within which amylose and  $\alpha$ -glucan determinations were duplicated.

Size Class	Mean Diameter ( $\mu$ m)	Age in days post-pollination					
		15	20	25	30	35	40
1	<0.8	4 $\pm$ 5	10 $\pm$ 4.7	8 $\pm$ 4.5	8 $\pm$ 3.7	6 $\pm$ 5	1 $\pm$ 0.9
2	1.4	5 $\pm$ 1.5	10 $\pm$ 1.0	15 $\pm$ 3.0	11 $\pm$ 0.9	10 $\pm$ 0.33	4 $\pm$ 0.9
3	2.0	12 $\pm$ 3.7	14 $\pm$ 2.4	15 $\pm$ 2.0	13 $\pm$ 0.3	11 $\pm$ 1.0	9 $\pm$ 3.7
4	3.2	12 $\pm$ 3.7	19 $\pm$ 3.4	20 $\pm$ 2.9	17 $\pm$ 8.5	16 $\pm$ 0.33	8 $\pm$ 3.2
5	4.8	16 $\pm$ 3.6	16 $\pm$ 3.1	20 $\pm$ 2.6	19 $\pm$ 4.0	16 $\pm$ 2.0	10 $\pm$ 2.3
6	7.2	21 $\pm$ 1.0	20 $\pm$ 3.2	22 $\pm$ 2.0	22 $\pm$ 2.0	21 $\pm$ 0.33	16 $\pm$ 0.3
7	>10.0	N.F. <sup>1)</sup>	19 $\pm$ 3.7	24 $\pm$ 2.0	21 $\pm$ 2.9	22 $\pm$ 1.66	18 $\pm$ 2.1

1) No fraction. Insufficient numbers were available in this size class for amylose determination.

6 there was a significant decrease in amylose percentage between 35 and 40 days post-pollination.

At every age examined the greatest percentage of total amyloplast numbers is in size class 2 (Table 3). The maximum is at 20 days and the minimum at 40 days. In classes 6 and 7 relative numbers increase steadily over the entire developmental period, together accounting for 0.7% of the total at 15 days, and for 7.8% of the total at 40 days. With the exception of size class 2, all classes increased significantly in percentage of total numbers between 15 and 40 days post-pollination. The total number of amyloplasts per unfractionated amyloplast pellet increased until 30 days falling off thereafter to a

steady value of 11.4 and 10<sup>8</sup> amyloplasts on an per endosperm basis.

The amount of  $\alpha$ -glucan present in classes 1 and 2 declined steadily throughout development (Table 4) apart from an initial increase at 20 days in class 2. The overall effect in classes 3, 4, and 5 was a decrease whereas, in class 6, an initial pronounced increase was followed by little change. In class 7 there was a rapid increase in  $\alpha$ -glucan content throughout development. It is noteworthy that, at 15 days post-pollination, over 60% of total  $\alpha$ -glucan was contained in classes 1 to 4 whereas, at 40 days post-pollination, 70% was associated with classes 5 to 7.

Table 3.  
Percentage of Total Number of Amyloplasts in Each Size Class During Endosperm Development in Sweet Corn. Unfractionated amyloplast pellets were counted (see Materials and Methods). Results for each stage of development are the mean of four samples  $\pm$  standard deviation. Each sample contained around  $2 \times 10^3$  amyloplasts.

Size Class	Diameter ( $\mu$ m)	Age in days post-pollination					
		15	20	25	30	35	40
1	<0.8	12.2 $\pm$ 0.4	8.5 $\pm$ 0.8	11.6 $\pm$ 1.3	10.5 $\pm$ 2.5	14.0 $\pm$ 1.3	15.8 $\pm$ 2.9
2	1.4	58.0 $\pm$ 3.7	70.0 $\pm$ 3.4	65.0 $\pm$ 2.2	60.0 $\pm$ 6.2	37.8 $\pm$ 1.7	33.6 $\pm$ 2.7
3	2.0	14.0 $\pm$ 2.7	9.7 $\pm$ 1.4	9.7 $\pm$ 0.6	14.4 $\pm$ 1.4	20.5 $\pm$ 2.5	19.4 $\pm$ 1.1
4	3.2	12.1 $\pm$ 1.6	7.2 $\pm$ 1.0	9.0 $\pm$ 0.7	7.8 $\pm$ 0.8	16.2 $\pm$ 2.4	18.4 $\pm$ 1.4
5	4.8	3.0 $\pm$ 0.5	2.6 $\pm$ 0.2	2.5 $\pm$ 0.6	3.8 $\pm$ 1.0	6.2 $\pm$ 1.1	5.0 $\pm$ 0.8
6	7.2	0.6 $\pm$ 0.3	1.3 $\pm$ 0.1	1.3 $\pm$ 0.3	2.0 $\pm$ 0.9	2.8 $\pm$ 0.5	3.3 $\pm$ 0.3
7	>10.0	0.1 $\pm$ 0.2	0.4 $\pm$ 0.1	0.9 $\pm$ 0.3	1.5 $\pm$ 0.2	2.5 $\pm$ 0.5	4.5 $\pm$ 1.2
No. of amyloplasts (pellet) endosperm ( $\times 10^{-8}$ )		3.4 $\pm$ 0.11	8.8 $\pm$ 0.08	13.1 $\pm$ 0.07	15.2 $\pm$ 0.17	11.4 $\pm$ 0.10	11.4 $\pm$ 0.09

Table 4.  
Percentage of Total Amyloplast  $\alpha$ -glucan at Each Age Associated with Each Size Class During Endosperm Development in Sweet Corn. Amyloplasts in each size range (see Materials and Methods) were washed twice in ice-cold distilled water. The results were calculated from values for the total numbers and  $\alpha$ -glucan content of amyloplasts present in each size range. A minimum of  $9 \times 10^3$  amyloplasts was counted in size classes 0.8 to 2.0  $\mu$ m,  $1.7 \times 10^3$  in size class 3.2  $\mu$ m and  $3 \times 10^3$  in size classes 4.8 to >10  $\mu$ m. In order to obtain the required numbers in the larger sizes at least four samples were counted in each case. Results are averages of two separate experiments within which starch determinations were duplicated.

Size Class	Diameter ( $\mu$ m)	Age in days post-pollination					
		15	20	25	30	35	40
1	<0.8	2.4	1.7	2.0	1.4	1.2	1.2
2	1.4	11.9	14.2	11.8	8.4	3.4	2.6
3	2.0	7.6	5.5	4.8	5.6	5.2	4.2
4	3.2	39.6	23.5	26.2	17.6	23.3	21.7
5	4.8	25.8	22.3	18.9	22.0	23.3	16.3
6	7.2	12.0	26.0	22.8	27.4	24.7	25.0
7	>10.0	1.7	6.8	13.5	17.6	18.9	29.0

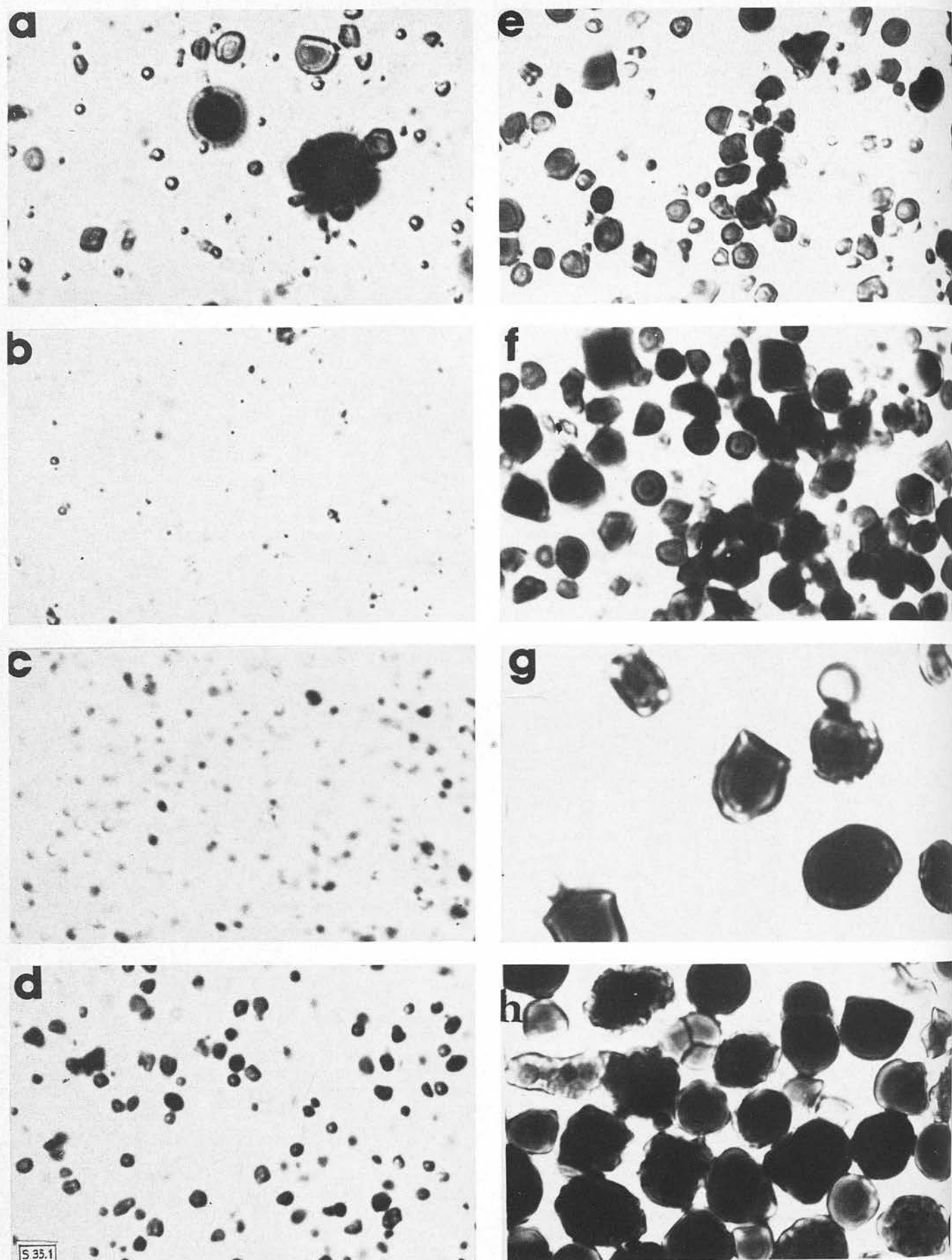


Figure 1. Light photomicrographs of sweet corn endosperm amyloplasts, at 30 days post-pollination, before and after separation on sucrose density gradients: a = unfractionated amyloplast pellet; b = size class 1; c = size class 2; d = size class 3; e = size class 4; f = size class 5; g = size class 6; h = size class 7 (Magnification  $\approx \times 1,500$ ).

That amyloplasts are present in a wide range of different sizes in immature maize endosperm is shown in Figure 1a. Representative fractions of amyloplasts in the different size classes from endosperm 30 days post-pollination, are also shown in Figure 1. The sizes within a given class are remarkably similar, with the exception perhaps of class 7 (Fig. 1h).

Since the fractions were not examined by electron microscopy for the presence of a surrounding double membrane, doubt must remain as to the relative numbers of amyloplasts and granules. However, since the methods used were mild and omitted any purification steps designed to remove lipid and protein it is likely that a high proportion of amyloplasts is present. This observation is borne out by the presence of large numbers of intact compound amyloplasts and phytoglycogen globules visible in size class 7. However, while the fractions are termed "amyloplast fractions" it is possible that there may be some contamination with granules and granule fragments.

## 4 Discussion

The increase in amylose content of unfractionated, twice washed sweet corn amyloplasts was similar to that observed previously by *Badenhuizen* [3] and *Boyer* [4]. It is thus clear that for maize as well as wheat [2] and barley [1] this is a characteristic of grain maturation. As with immature [6] and mature barley [12] and maize [4] amyloplasts, the amylose content of the smaller amyloplasts was found to be less than that of the larger. This is not surprising, at least at early stages of development, since the small amyloplasts are presumably synthesised first and therefore account for the major amount of polysaccharide present at that time. The polysaccharide laid down initially in sweet corn amyloplasts may then be either amylopectin or phytoglycogen since it has been reported [7] that the very small granules of sweet corn endosperm contain some phytoglycogen. It is clear that in the present case the increase in amylose content is not caused by increasing numbers of amyloplasts all of the same composition but rather by different rates of appearance of amyloplasts of differing composition. That the rates of appearance must differ may be seen from the results of Tables 1 and 2, where at 15 days an overall amylose content of  $10\% \pm 1.8$  must be made up of relatively more of size classes 1 to 4, whereas at 35 days, for example, an overall amylose content of  $23\% \pm 1.8$  must be made up of relatively more of size classes 6 and 7. That this is, in fact, the case is demonstrated in Tables 3 and 4 where at 15 days 61.5% of total amyloplast  $\alpha$ -glucan contained in 96% of the total number of amyloplasts is associated with size classes 1 to 4. On the other hand at 40 days over 70% of total  $\alpha$ -glucan contained in 12.5% of total endosperm amyloplasts is in size classes 5 to 7.

The greatest change in amylose percentage within a given size class is restricted to the smaller amyloplasts, and, in particular to class 2. The larger amyloplasts change little in  $\alpha$ -glucan composition throughout, suggesting that, once synthesised, they are not subject to intermediary degradation. The smallest amyloplasts (classes 1 and 2), however, contribute at most, only 16% of the total  $\alpha$ -glucan to the whole, and so it is likely that the change in amylose content with age is mainly due to differences in the relative numbers of the different size classes present. It is worth noting that while there is an increase in the percentage of amyloplasts in size classes 3 and 4 between 20 and 40 days post-pollination, this is accompanied by a fall in total  $\alpha$ -glucan in size class 3 and little change in size

class 4. This means that the amount of  $\alpha$ -glucan per amyloplast decreases with time, the size remaining the same. This is not the case with the other size classes. Thus, some of this polysaccharide must be used, either in respiratory processes which are active during grain development [13], or in deposition of carbohydrate in the larger amyloplasts. Other results (not shown) indicate that the total number of smaller amyloplasts decreases by 75% between 25 and 40 days post-pollination. This, as we see here, is accompanied by a marked decrease in  $\alpha$ -glucan content. Thus, the polysaccharide from those originally present must also be respired or appear later in the large amyloplasts. In maize, if not in barley [9] where it may be that small granules are synthesized at a discrete time (14 days) after anthesis — the small granules at later stages may be derived from physiologically younger cells [14]. The present results suggest that either such synthesis decreases by 25 to 30 days post-pollination or that their turnover and degradation is an extremely rapid process. Certainly the total numbers of amyloplasts present decrease significantly after 30 days indicating that initiation of new organelles may have decreased or halted by this stage. At the same time polysaccharide continues to accumulate, but in rather fewer amyloplasts of larger than average size.

In conclusion, the increase in percentage amylose content of amyloplast  $\alpha$ -glucan during sweet corn kernel maturation is due to changes in relative numbers of the different size classes present, each of which shows increasing amylose content with increasing size throughout development, and to changes during this period in the amylose content of size classes 1 and 2.

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# Oxygen Exchange in the Pericarp Green Layer of Immature Cereal Grains<sup>1</sup>

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## ABSTRACT

Rates of oxygen exchange in light and dark were recorded for immature detached barley spikelets and wheat florets both before and after successive removal of the husk (palea and lemma), transparent layer of the pericarp, and green layer of the pericarp. Results were compared with those for the mutant barley *Albino lemma* which has a pericarp lacking chlorophyll. There was no net oxygen evolution in the intact spikelets of *Albino lemma* when incubated in the light. Removal of the husk increased the rate of measured oxygen uptake in both light and dark. With normal barley and wheat, net oxygen evolution in the light was observed in intact spikelets and florets, as well as after husk removal and after both husk and transparent layer removal. Additional removal of the green layer of the pericarp resulted in a dramatic changeover from oxygen evolution in the light to oxygen uptake. The results suggest that some of the oxygen generated by pericarp photosynthesis remains within the grain.

The dry matter entering the barley grain is primarily derived from photosynthesis occurring after ear emergence (1), the ear itself contributing up to 76% of the total CO<sub>2</sub> fixed (4). In wheat all of the separated parts of the immature ear, including outer glumes, lemmas, paleae, rachis, and grains, are capable of light-dependent CO<sub>2</sub> fixation (3). The separated grain can account for about 40% of gross photosynthesis and this is presumably derived from CO<sub>2</sub> fixation in the Chl-containing layers of the pericarp. During most of the grain-filling period the pericarp is a bright emerald green tissue surrounded by a transparent outer layer. Previous work (2, 5) with barley has shown that the green layer is capable of photosynthesis and that a number of enzymes normally associated with photosynthetic CO<sub>2</sub> fixation are present. These include ribulose bisP carboxylase and P-enolpyruvate carboxylase. Thus, although the pericarp is capable of CO<sub>2</sub> fixation the significance of its location within the tissues of the ear is not clear.

The aim of the present work was to investigate O<sub>2</sub> exchange in the green layer of the pericarp in immature cereal grains by comparing results obtained in barley spikelets and wheat florets with those obtained under the same conditions for a mutant variety of barley, *Albino lemma*, which lacks Chl in the pericarp. O<sub>2</sub> exchange measurements were selected as a convenient measure of photosynthesis, which in the dark, would additionally give an indication of respiratory activity.

## MATERIALS AND METHODS

**Plant Material.** The barleys, *Hordeum distichum* (L.) Lam. var. *Albino lemma* and *Hordeum vulgare* (L.) var. *Albino lemma* and the wheat,

*Triticum aestivum* (L.) var. Maris Dove, were grown in greenhouses with natural daylength extended to 18 hr with mercury vapor lamps. All plants were grown in soil at a density of six plants/pot (18-cm diameter).

**Measurement of O<sub>2</sub> Exchange.** Three spikelets or florets were placed in the chamber of a Rank oxygen electrode with 3.0 ml of a solution containing 50 mM Tricine-KOH (pH 7.5), 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, and 330 mM sorbitol, and agitated at constant speed using a magnetic stirrer. Samples were selected from halfway up the ear where growth conditions are similar and variation in weight minimal. The chamber was jacketed by a circulating water supply at 30 C. Initial rates of O<sub>2</sub> exchange were measured in the dark using an aluminum foil shade, when the system had reached steady-state conditions. Afterwards the shade was removed and the chamber illuminated with a 275-w (pearl) Phillips No. 1 Photoflood tungsten lamp, the bulb of which was 15 cm from the center of the chamber. Illuminance within the chamber was 658  $\mu\text{E m}^{-2} \text{sec}^{-1}$  (400-700 nm).

The rates of O<sub>2</sub> uptake in the dark and in the light were assumed to be the same. This is certainly true for the transparent layer and the grain when stripped of all photosynthetic tissue. On the other hand, the presence of photorespiration in green tissue would result in an increased O<sub>2</sub> uptake in the light compared to that in the dark. It may be, however, that photorespiration is low in the green layer of the pericarp since ribulose bisP carboxylase activity is low compared to that of P-enolpyruvate carboxylase (2). Glycolate oxidase activity, an additional marker of photorespiration, is also low compared to that found in barley and wheat leaves (A. R. Nutbeam, in preparation).

Each experiment with three grains was conducted three times and the mean of the three rates calculated. The rate of O<sub>2</sub> exchange was expressed in nmol of O<sub>2</sub>/min·grain or grain part. Rates of O<sub>2</sub> exchange were also recorded for wheat florets and barley spikelets after successive removal of: (a) the husk (palea and lemma); (b) the transparent layer of the pericarp; and (c) the green layer of the pericarp. As *Albino lemma* pericarp has no green layer the final stage of layer removal was necessarily omitted.

Light-dependent O<sub>2</sub> evolution was also measured in isolated husks and green layers of pericarp.

## RESULTS

Net O<sub>2</sub> evolution in the light was observed in intact wheat florets and after successive removal of the husk and transparent layer of pericarp (Table I). Only when the pericarp green layer was removed was there a dramatic changeover from O<sub>2</sub> evolution to O<sub>2</sub> uptake. This figure ( $-11 \pm 1.0$  nmol/min·grain) was not significantly different from that ( $-13.3 \pm 1.0$  nmol/min·grain), observed in the dark. Removal of the husk caused an increase in light-dependent O<sub>2</sub> evolution from 10 to 18 nmol of O<sub>2</sub>/min·grain. The isolated husk had a small but significant rate of O<sub>2</sub> evolution in the light (2.0 nmol/min·grain) which, when corrected for O<sub>2</sub> uptake in the dark, resulted in 6 nmol/min·grain for light-de-

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pendent  $O_2$  evolution. Removal of the transparent layer of cells covering the pericarp green layer did not significantly change the rates of either  $O_2$  evolution in the light or light-dependent  $O_2$  evolution. However, it did result in a large increase in  $O_2$  uptake in the dark.

Corresponding data for barley are shown in Table II. Spikelets (minus awns), spikelets with the husk removed, and with the husk and the pericarp transparent layer removed showed net  $O_2$  evolution in the light. Successive removal of the husk and the transparent layer resulted in a sharp increase in  $O_2$  uptake in the dark.  $O_2$  evolution in the light was not significantly affected. Removal of the pericarp green layer resulted in a decrease in  $O_2$  uptake in the dark and a dramatic changeover from  $O_2$  evolution to  $O_2$  uptake, in the light. In these experiments  $O_2$  evolution in the light was not observed with isolated husks or pericarps. Taking into account  $O_2$  uptake in the dark, both tissues were capable of light-dependent  $O_2$  evolution.

Data for the barley *Albino lemma* are shown in Table III. No net  $O_2$  evolution in the light was obtained in any grain or grain fraction examined. Successive removal of the husk and transparent layer markedly increased the rate of  $O_2$  uptake in both light and dark. Both intact grains and isolated husks were capable of small but significant rates of light-dependent  $O_2$  evolution. Very low values for light-dependent  $O_2$  evolution in the grains with husk

Table III. Oxygen exchange in barley (*Albino lemma*)

Conditions are as described in Table II. Data are the mean of three estimates  $\pm$  standard deviation and are expressed in nmol  $O_2$ /min/spikelet or tissue fraction.  
+....  $O_2$  evolution; -....  $O_2$  uptake

Tissue	Oxygen exchange		
	Light	Dark	Light dependent $O_2$ exchange
Spikelet	- 0.9 $\pm$ 0.4	- 3.4 $\pm$ 0.8	+ 2.9 $\pm$ 0.3
Husk removed	- 6.3 $\pm$ 0.6	- 7.2 $\pm$ 0.3	+ 1.0 $\pm$ 0.6
Transparent layer of pericarp removed	- 6.6 $\pm$ 0.3	- 8.4 $\pm$ 0.4	+ 1.8 $\pm$ 0.6
Isolated husk	- 1.1 $\pm$ 0.1	- 3.4 $\pm$ 0.3	+ 2.3 $\pm$ 0.3

removed and with both husk and transparent layer removed were observed.

## DISCUSSION

In *Albino lemma* both in light and dark and in Julia in the dark, removal of the palea and lemma resulted in a marked increase in  $O_2$  uptake. This suggests that these outer layers may limit the influx of atmospheric  $O_2$ . In wheat the results were less marked and in the dark the slight increase in  $O_2$  uptake resulting from the removal of the husk indicated that in this case the palea and lemma may have been less of a barrier to  $O_2$  influx than in barley. The increase in light-dependent  $O_2$  evolution upon removal of the palea and lemma, which was greater in wheat than in barley, suggests either that these tissue constitute a barrier to  $O_2$  efflux from the green layer of pericarp or that the increased incident light resulted in higher rates of photosynthesis and hence of  $O_2$  evolution or that both factors contributed to the effect. Certainly, if the outer layers were entirely permeable, the respiration rate would be expected to fall on their removal as these tissues would no longer be present to add their  $O_2$  consumption to the total. In the case of *Albino lemma* the rates of  $O_2$  uptake in intact grains were similar to those in the isolated palea and lemma, suggesting that rates of  $O_2$  evolution measured for intact grains might be entirely accounted for by these tissues. This was not so clear cut in Julia or Maris Dove. If  $O_2$  access to the grain is restricted by the outer layers then the measured rates of respiration may not reflect *in vivo* rates since  $O_2$  levels may be limiting. In addition, if  $CO_2$  efflux is simultaneously prevented then, in the absence of internal refixation, its concentration will rise until respiration is reduced by lack of  $O_2$ .

Since removal of the transparent layer of the pericarp increased the rates of respiration markedly in both wheat and Julia and rather less in *Albino lemma* it is likely that this tissue may also be a barrier to  $O_2$  uptake. At the same time, rates of light-dependent  $O_2$  evolution increased. Thus, this layer may have properties similar to those described above for the palea and lemma.

Removal of the green layer of pericarp in wheat and barley resulted in similar rates of  $O_2$  uptake in both light and dark. It can therefore be concluded that this layer is responsible for the observed rate of light-dependent  $O_2$  evolution by isolated dehusked spikelets or florets. Since the green layer of pericarp dehydrates extremely rapidly on removal from the grain it was perhaps not surprising to observe no net  $O_2$  evolution in the light using isolated pericarps. The observed  $O_2$  uptake was presumably due to active respiration in the presence of nonlimiting supplies of  $O_2$ . While some damage inevitably occurred during spikelet and floret removal, little or no apparent dehydration of endosperm or outer layers was observed during the course of the experiment.

The results suggest that some of the  $O_2$  generated by pericarp photosynthesis remains within the grain. The source of  $O_2$  for endosperm respiratory processes may thus be the pericarp rather than the outer atmosphere.

Table I. Oxygen exchange in wheat var. Maris Dove

Wheat florets were removed at the stage of development most closely corresponding to that of the barley spikelet at 25 days after anthesis. The tissue used for analysis is that remaining after successive removal of the outer layers described below. Data are the mean of three estimates  $\pm$  standard deviation and are expressed in nmol  $O_2$ /min/floret or tissue fraction.  
+....  $O_2$  evolution; -....  $O_2$  uptake

Tissue	Oxygen exchange		
	Light	Dark	Light dependent $O_2$ evolution
Floret	+ 3.0 $\pm$ 0.3	- 7.0 $\pm$ 0.0	+ 10.0 $\pm$ 0.3
Palea and lemma removed	+ 9.0 $\pm$ 2.0	- 9.0 $\pm$ 0.3	+ 18.0 $\pm$ 1.0
Transparent layer of pericarp removed	+ 5.0 $\pm$ 3.0	- 16.0 $\pm$ 2.0	+ 21.0 $\pm$ 4.0
Green layer of pericarp removed	- 11.0 $\pm$ 1.0	- 13.3 $\pm$ 1.0	+ 3.0 $\pm$ 2.0
Isolated husk	+ 2.0 $\pm$ 1.0	- 4.0 $\pm$ 0.3	+ 6.3 $\pm$ 1.0

Table II. Oxygen exchange in barley var. Julia

Spikelets were removed at 25 days after anthesis. The total period between anthesis and full maturity was 60 days. Awns were removed as these were too long to fit in the chamber of the oxygen electrode. The tissue used for analysis is that remaining after successive removal of the outer layers described below. Data are the mean of three estimates  $\pm$  standard deviation and are expressed in nmol  $O_2$ /min/spikelet or tissue fraction. +....  $O_2$  evolution; -....  $O_2$  uptake.

Tissue	Oxygen exchange		
	Light	Dark	Light dependent $O_2$ evolution
Spikelet	+ 5.4 $\pm$ 1.6	- 3.8 $\pm$ 0.8	+ 9.4 $\pm$ 1.1
Husk removed	+ 4.3 $\pm$ 2.9	- 8.2 $\pm$ 0.6	+ 12.5 $\pm$ 3.4
Transparent layer of pericarp removed	+ 1.2 $\pm$ 3.5	- 13.3 $\pm$ 0.6	+ 17.3 $\pm$ 1.7
Green layer of pericarp removed	- 8.4 $\pm$ 0.2	- 10.3 $\pm$ 0.5	+ 1.8 $\pm$ 0.2
Isolated husk	- 1.0 $\pm$ 0.4	- 6.2 $\pm$ 1.4	+ 5.3 $\pm$ 1.8
Isolated green layer of pericarp	- 2.3 $\pm$ 0.3	- 2.8 $\pm$ 0.2	+ 0.5 $\pm$ 0.0

*Acknowledgment* We wish to thank M. T. D. Carr for his helpful discussions.

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## Sucrose Transport in Isolated Immature Barley Embryos

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### ABSTRACT

Techniques have been devised to select immature barley embryos at various stages in their development, and to study their accumulation of sucrose *in vitro*. Isolated embryos accumulate sucrose over a period of several hours of which some 80 per cent is conserved as a pool of free sucrose and the remainder utilized in macromolecular synthesis. The rate of sucrose uptake increases with embryo development, however the specific activity of uptake remains constant, indicating that the transport processes are fully operative early in embryogenesis. From the kinetics of sucrose uptake it is deduced that facilitated transport predominates at sucrose concentrations of 50 mM, while at higher concentrations passive diffusion makes an increasing contribution to sucrose accumulation. The substrate specificity and the sensitivity of sucrose transport to uncoupling agents, in addition to the stability of the pool of accumulated sucrose, are all indicative of active transport playing a major role in the sucrose assimilation of developing barley embryos.

Key words: *Hordeum distichum* (L.) Lam, barley, embryo, sucrose transport.

### INTRODUCTION

A better understanding of the relationship between the developing barley embryo and its environment has become increasingly important with the desire of plant breeders to speed mutant selection with the use of *in vitro* embryo culture (Jensen, 1976). A study of tissue culture media best suited for the *in vitro* culture of barley pro-embryos (Cameron-Mills and Duffus, 1977), revealed that the embryos are exacting in their nutritional requirements. In common with many other plant embryos, the only carbohydrate source which supported normal development and germination was sucrose (Narayanaswami and Norstog, 1964).

It is well established that sugars entering the developing grain are derived from photosynthesis after ear emergence (Archbold, 1942), to which photosynthesis by the ear itself together with the flag leaf, make the major contribution (Frey-Wyssling and Buttrose, 1959). While sucrose has been identified as a major component of the translocation stream, our knowledge of the composition of the low-molecular-weight sugars in the developing grain is far from detailed. During the period of rapid embryo growth, 20-40 days after anthesis (Duffus and Rosie, 1975), the total free sugars comprise between 2 and 3 per cent of the grain d. wt (LaBerge, MacGregor and Meredith, 1973). While reducing sugars, in particular glucose and fructose, form an important part of this fraction early in grain development, sucrose as the predominant non-reducing sugar can account for 80-90 per cent of the soluble sugars present during the major developmental phase.

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There is evidence that the composition and concentration of nutrients in the developing grain is subject to regulation. In wheat ears at least, this appears to depend on the regulation of translocation between the rachis and the phloem in the grain furrow (Jenner, 1976). Thus, *in vivo* and *in vitro*, the maximum sucrose concentration found in the wheat endosperm is 58 mM. This is maintained uninfluenced by diurnal variations in the photosynthetic organs. It has also been observed that under adverse growth conditions and limiting photosynthate, the optimal nutrient levels for grain development are conserved by initiating the development of fewer spikelet primordia (Scott, 1976). It appears that embryo development may be constrained by the supply of translocated material. Certainly, where the nutrient supply is unlimited during *in vitro* culture, the rate of embryo growth is significantly enhanced (Cameron-Mills and Duffus, 1977).

This paper presents the results of an investigation into the efficiency of sucrose assimilation by developing barley embryos, with particular emphasis on the kinetics and energetics of the transport process.

#### MATERIALS AND METHODS

The two-row barley varieties *Hordeum distichum* (L.) Lam. cv. Julia and Midas were grown in a greenhouse at approximately 20 °C under natural light, extending the day-length to 18 h with Osram 400 W sodium lamps. The first six tillers of any plant were labelled on the day of anthesis, as described by Merritt and Walker (1969) and the ears removed at various stages of development. Twelve grains from the central portion of each ear were dehusked and sterilized in 5 per cent sodium hypochlorite for 10 min and thoroughly rinsed in sterile distilled water. The excision of embryos and subsequent transport assays were performed under sterile conditions. Since anthesis dating is subject to a 1- to 2-day error and the main embryo growth period is only 10 days, the final selection of barley ears was based on the size of the isolated embryos. From each selected ear one embryo was placed in each sample tube and stored on ice in 5 mM phosphate buffer pH 5.0 prior to assay.

Unless otherwise specified, embryos were incubated in 1 ml of medium containing 5 mM phosphate buffer pH 5.0 and 100 mM [ $U-^{14}C$ ]sucrose (specific activity 1  $\mu$ Ci 100  $\mu$ mol<sup>-1</sup>) at 25 °C in a shaking water bath to determine their uptake of sucrose. To terminate each assay, the embryos were collected by Millipore filtration and washed in non-radioactive medium. The 80 per cent ethanol soluble sugars were extracted at 70 °C with three successive volumes of solvent and the pooled extracts were dried down under vacuum. After re-solubilization in 80 per cent ethanol, the samples were pipetted onto glass-fibre discs which were dried and suspended in toluene-2,5-diphenyl oxazole-1,4-bis(5 phenyloxazol-2-yl) benzene scintillant for radioactivity measurements in a Beckman LS 3155T scintillation spectrometer. Radioactivity remaining in the embryo 80 per cent ethanol-insoluble fraction was determined with the aid of a Packard Tricarb Sample Oxidiser 306.

Chromatographic separation of sugars was performed on Whatman No. 1 paper in a descending system for 18 h in a solvent of ethyl acetate:pyridine:water (10:4:3 v/v/v). The 80 per cent ethanol extract from 1 mg embryo dry weight was co-chromatographed with 20  $\mu$ g each of the following marker sugars: rhamnose, xylose, mannose, fructose, glucose, galactose, maltose, sucrose and raffinose. Mono-, di- and oligosaccharides were detected with a non-specific silver nitrate dip (Trevelyan, Procter and Harrison, 1950). The monosaccharides were distinguished with an anisidine spray reagent (Pridham, 1956) and the phosphorylated sugars with an ammonium molybdate reagent (Hanes and Isherwood, 1949). Kodak materials were used for autoradiography of the paper chromatograms.

## RESULTS

*Parameters of embryo growth*

One of the most important aspects of experimental design in the study of developing embryos is the selection of plant material. While the ageing of barley ears from the time of anthesis may provide a valuable criterion for the initial selection of material, the rate of embryo development is strongly influenced by their position in the ear. Embryos isolated from a 15-day ear were found to range in length between 1.84 and 2.25 mm, which is substantial in a selected developmental period of 1.0–3.2 mm size embryos. Since embryo development is dominated by growth in a single plane, measurement of embryo length, namely scutellum perimeter to coleorhiza tip, provided a convenient parameter of embryo age.

In order to obtain a more critical comparison of embryos at different developmental stages it was necessary to express the results both in terms of numbers of embryos and embryo dry weight. The relationship between embryo length and embryo f. and d. wt is shown in Fig. 1(a), (b), and when plotted on semi-log paper (not shown) the relationship is found to be linear over a large portion of embryogenesis. While barley embryos attain a maximum size of approximately 3.5 mm in length the continued deposition of storage material allows for significant further increases in weight. For this reason the developmental period selected for study was limited to those embryos still increasing in size. Since the embryo size in all sucrose assimilation studies was monitored, the d. wt of plant material could be derived from the embryo length/embryo weight correlation plots, thereby avoiding the problems of weighing small numbers of embryos.

While the differences in embryo growth between Julia and Midas varieties must reflect variations in morphogenesis, the d. wt of their embryos at maturity (not shown) is very similar.

*Sucrose assimilation by immature barley embryos in vitro*

Sucrose uptake from the standard assay medium containing 100  $\mu\text{M}$  [ $\text{U-}^{14}\text{C}$ ]sucrose by immature embryos was determined over a period of 6 h. Sucrose was rapidly accumulated into the 80 per cent ethanol-soluble fraction of isolated embryos (Fig. 2) and after 6 h incubation there was no evidence of a decline in accumulation rate. In the absence of sucrose metabolism within the pool, the concentration of free sucrose at the end of the incubation would be 82  $\mu\text{g}$  per mg embryo d. wt.

*Storage of isolated embryos*

During the preparation of material for each experiment, isolated embryos were stored in ice-cold 5 mM phosphate buffer, pH 5.0. While this facilitated the further washing off of contaminating endosperm tissue from the embryos, the possible deleterious effects of prolonged storage on embryo sucrose accumulation had to be investigated.

Prolonged storage of isolated embryos in ice-cold buffer (Table 1) led to a progressive increase in their sucrose uptake capacity, which may have arisen from a gradual leakage or depletion of their free sucrose pool. However, provided the average embryo storage time of samples within each experiment were the same, this should not interfere with the interpretation of the data.

*Optimization of the sucrose accumulation assay*

The isolation of undamaged, immature embryos is a delicate and lengthy operation and, in a large-scale experiment, may result in the storage of embryos for as much as 4 h prior to assay. In order to reduce the storage period while maximising the scope of each

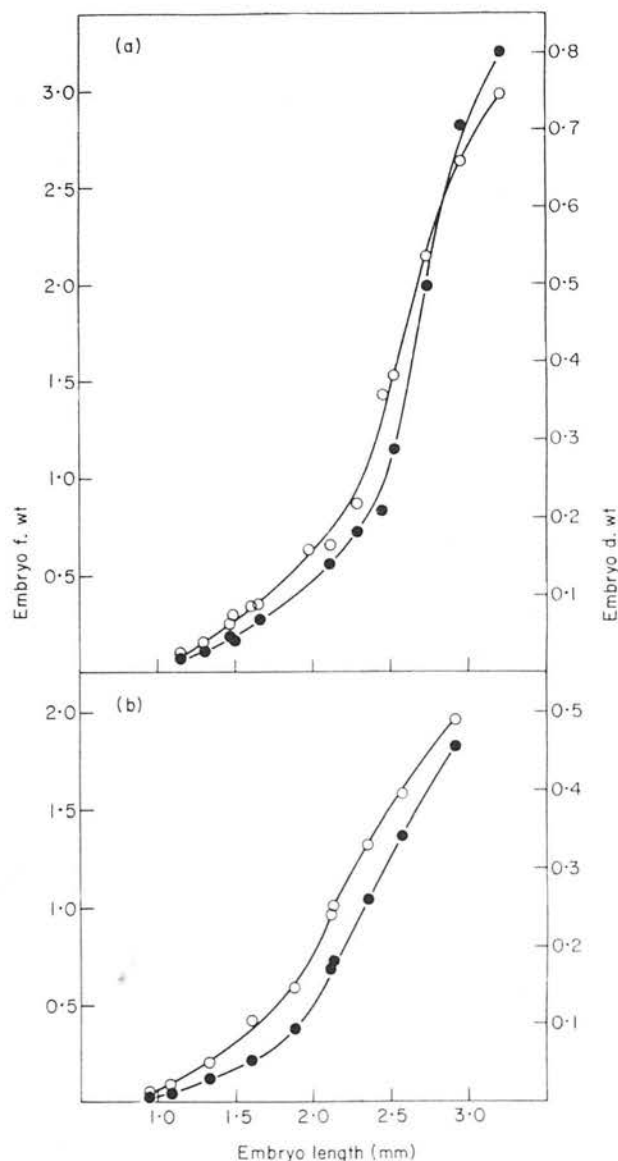


FIG. 1. Parameters of barley embryo growth. The relationship between the fresh (○) and dry weight (●) of isolated Midas (a) and Julia (b) embryos and their increase in length, as measured between the scutellum perimeter and the coleorhiza tip.

experiment, the least number of embryos per sample required to give reproducible results was determined. Seven embryos per sample were found satisfactory (Table 2) provided that the embryos from each selected ear were equally represented in all samples and the average storage time was standardized.

When studying carbohydrate metabolism in small samples of plant material during 25 °C incubations extending to several hours, the interference of microbial proliferation must be avoided. To prevent contamination the dehusked grains were sterilized in 5 per cent sodium hypochlorite, washed in sterile water and all subsequent steps were performed under sterile conditions. Comparisons of sterile and non-sterile embryos confirmed that

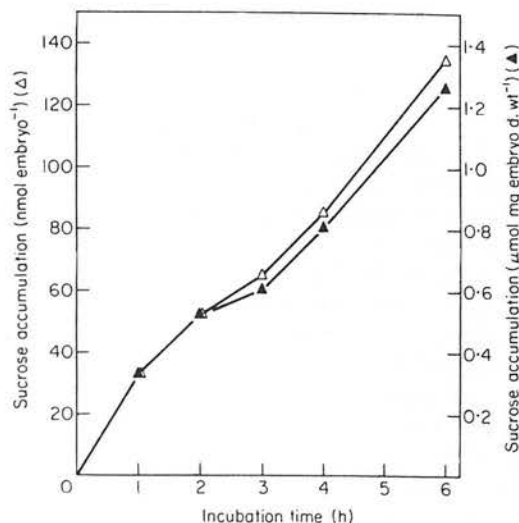


FIG. 2. Sucrose accumulation by immature barley embryos *in vitro*. Isolated Julia embryos (average length  $1.96 \pm 0.13$  mm) were incubated in 5 mM phosphate buffer pH 5.0 containing 100 mM [ $U\text{-}^{14}C$ ]sucrose (specific activity  $1 \mu\text{Ci } 100 \mu\text{mol}^{-1}$ ) at  $25^\circ\text{C}$  in a shaking water bath. Samples of embryos (7) were harvested at intervals and the accumulation of sucrose in the 80 per cent ethanol-soluble fraction determined.

TABLE 1. *In vitro* sucrose accumulation by stored immature barley embryos. Immature Midas embryos (average length  $1.62 \pm 0.13$  mm) were stored in ice-cold 5 mM phosphate buffer, pH 5, and their subsequent sucrose uptake capacity determined in the standard assay medium and compared with that of freshly isolated embryos

Storage time (min)	Sucrose accumulation rate $\text{nmol h}^{-1} \text{ mg embryo d. wt}^{-1}$
0	150
15	156
30	158
60	192
120	200
240	289

TABLE 2. *Reproducibility of in vitro sucrose accumulation by immature barley embryos. The initial rate of sucrose uptake by immature Midas embryos was determined in the standard assay medium as described in Materials and Methods*

Average embryo length (mm)	Sucrose accumulation rate	
	$\text{nmol h}^{-1} \text{ embryo}^{-1}$	$\text{nmol h}^{-1} \text{ mg embryo d. wt}^{-1}$
2.89	66.3	102
2.88	63.4	99
2.95	67.7	97
2.86	66.2	107
2.90	61.5	95
2.88	64.7	101
Mean $\pm$ s.e.	$65 \pm 2$	$100 \pm 4$

these procedures had no deleterious effect on the carbohydrate accumulation of isolated embryos.

#### *Characterization of sucrose accumulation*

The capacity of immature embryos to accumulate sucrose at various stages of development was determined and found to increase with the growth of the embryo (Fig. 3). By comparing initial rates of sucrose uptake over a range of substrate concentrations, the kinetics of uptake in embryos at various stages of development could be compared. When the external sucrose concentration was raised from 5 to 100 mM, the initial sucrose uptake rate in embryos at each stage of development increased, the response being greatest in the older embryos [Fig. 4(a)]. However, although an increased capacity to accumulate sucrose accompanies embryo development, the specific activity of the process, when expressed as  $\text{nmol sucrose h}^{-1} \text{mg embryo d. wt}^{-1}$ , remains fairly constant [Fig. 4(b)]. When the data in Fig. 4(b) is replotted in a reciprocal form [Fig. 4(c)], it can be seen that sucrose uptake by embryos, throughout this developmental phase, observes saturation kinetics with a  $K_m$  of 36 mM and a  $V_{\max}$  of  $227 \text{ nmol h}^{-1} \text{mg}^{-1} \text{embryo d. wt}^{-1}$ . A deviation from the linear regression plot occurs at the highest substrate concentrations, indicating that the plot may be biphasic as a result of a non-saturable diffusion process contributing to sucrose uptake. This would also explain why the  $V_{\max}$  derived from the Lineweaver-Burke plot is significantly lower than that extrapolated from the Michaelis-Menten plot.

It may, however, be concluded that facilitated transport, involving a defined number of carrier sites, plays an important role in the sucrose accumulation of developing embryos *in vitro*.

#### *The fate of accumulated sucrose*

During the first 2 h incubation in the standard assay medium approximately 20 per cent of the sucrose accumulated by immature embryos is incorporated into macromolecular material, the remainder accumulating in an 80 per cent ethanol-soluble pool. The nature of the  $^{14}\text{C}$ -labelled material stored in this pool was investigated with the aid of paper chromatography and autoradiography. In freshly isolated embryos the sugars detected in the pool, at concentrations of  $5\text{--}10 \mu\text{g mg}^{-1} \text{embryo d. wt}$ , included glucose, fructose, xylose, sucrose, raffinose, various molecular weight fructans and sugar phosphates. After incubation with  $[\text{U-}^{14}\text{C}]\text{sucrose}$ , analysis of the embryos revealed a significant increase in detectable sucrose and autoradiography confirmed that this was the only  $^{14}\text{C}$ -labelled sugar in the pool. Incorporation of  $^{14}\text{C}$  label into non-carbohydrate compounds was very limited, since the radioactivity present in the sucrose spot accounted for 80 per cent of the  $^{14}\text{C}$ -labelled pool.

#### *Sensitivity of sucrose accumulation to metabolic inhibitors*

The effect of three uncoupling agents on sucrose uptake by immature embryos in the standard assay medium was determined over a period of 140 minutes (Table 3). Although at a concentration of 1 mM, sodium arsenate did not affect the rate of sucrose uptake, embryos were sensitive to both sodium azide and 2,4-DNP. While 0.01 mM 2,4-DNP was at a concentration sufficient to cause a significant inhibition of uptake, higher concentrations did not increase the inhibition.

While the kinetics of sucrose accumulation by immature embryos are characteristic of a facilitated transport mechanism, its sensitivity to uncoupling agents is indicative of an active transport process. However, from this result alone one could not deduce that the transport process itself is the primary active step in sucrose assimilation. The maintenance of a concentration gradient between the embryo and its environment, dependent on the further metabolism and removal of accumulated sugar, could be sufficient to drive the

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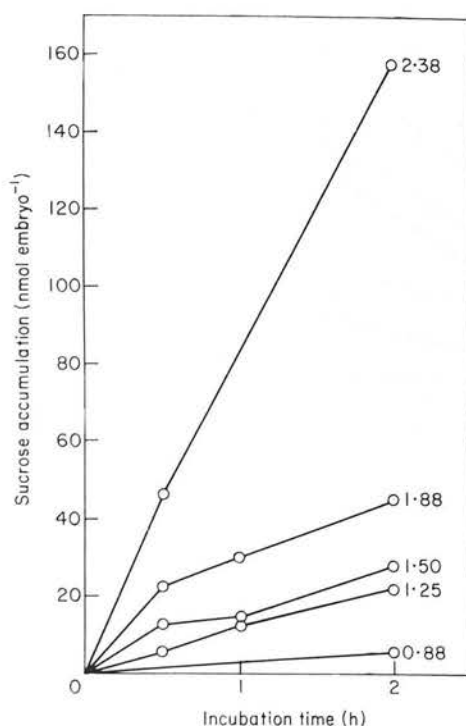


FIG. 3. The relationship between the development of barley embryos and their capacity to accumulate sucrose *in vitro*. The accumulation of sucrose *in vitro* by immature Julia embryos, isolated at various stages of development, was measured as described in Fig. 2. Embryo length (mm) is represented at the end of each graph line.

influx of external sucrose. This alternative interpretation is unlikely since it has already been shown that a major part of the accumulated sucrose is stored as sucrose in a pool.

#### Stability of the sucrose pool

The hypothesis that sucrose uptake by immature embryos *in vitro* is mediated by active transport would be further supported if it could be shown that its pool is stable in the presence of a concentration gradient. The stability of the pool was investigated in the following manner.

Immature embryos were pre-loaded with [U- $^{14}$ C]sucrose by incubation in the standard assay medium for 2 h. The embryos were then harvested and washed in unlabelled sucrose and buffer solution, and a sample of embryos removed to determine the amount of sucrose accumulated. The remaining embryos were incubated in buffer from which samples were removed after various intervals, to determine the [ $^{14}$ C]sucrose efflux rate. After 150 min incubation the embryos were harvested and the size of the 80 per cent ethanol-soluble and insoluble fractions measured (Fig. 5).

During the first incubation, sucrose was accumulated rapidly, of which 17 per cent was incorporated into macromolecular material. In the second incubation in buffer alone, macromolecular synthesis continued, drawing on a further 25 per cent of the [ $^{14}$ C]sucrose pool. Sucrose efflux from the pool, which occurred in the first 15 min after transfer into buffer, accounted for less than 5 per cent of the pool. At the end of the incubation 44 per cent of the accumulated sucrose pool remained and only 9 per cent had been consumed by respiratory metabolism.

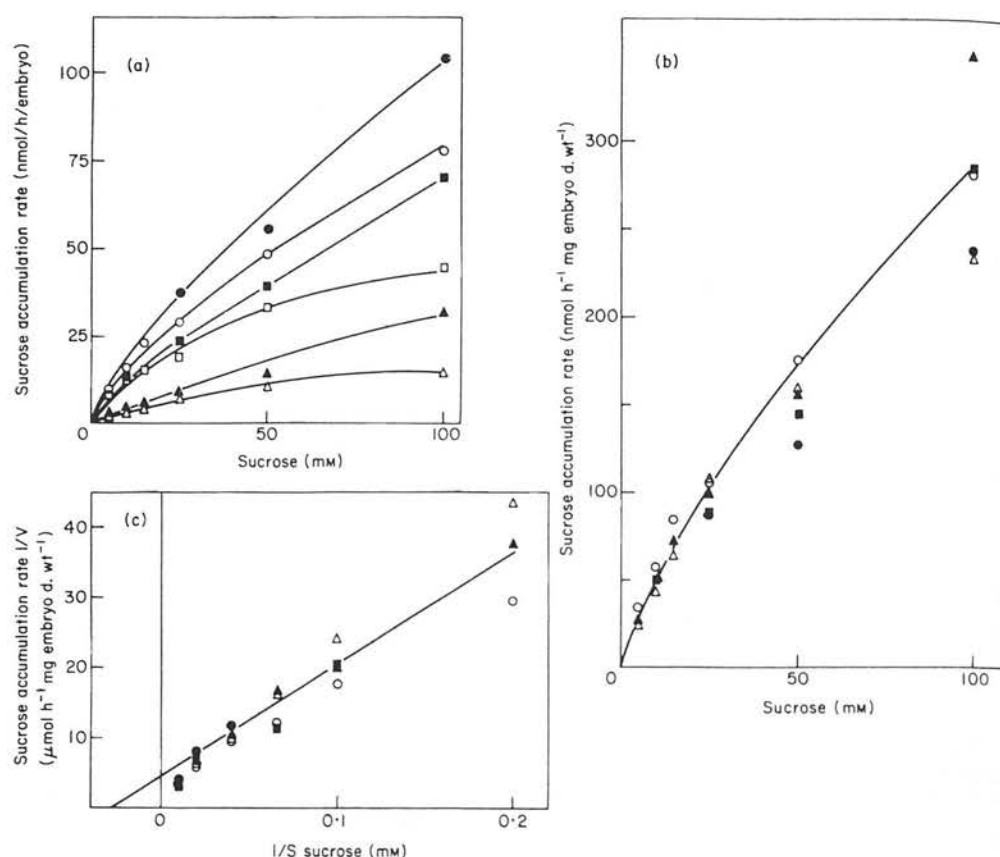


FIG. 4. The kinetics of sucrose accumulation *in vitro* by the developing barley embryo. The accumulation of sucrose *in vitro* by immature Julia embryos, isolated at various stages in their development, was measured at a range of substrate concentrations as described in Fig. 2. Rates of sucrose uptake are expressed per embryo (a) or per mg embryo d. wt. (b, c). The average embryo sizes were as follows:  $\triangle$ , 1.65 mm;  $\blacktriangle$ , 1.83 mm;  $\square$ , 2.06 mm;  $\blacksquare$ , 2.37 mm;  $\circ$ , 2.61 mm;  $\bullet$ , 2.87 mm ( $\pm 0.13$  mm).

TABLE 3. The effect of metabolic inhibitors on *in vitro* sucrose accumulation by immature barley embryos. The initial rate of sucrose uptake by immature Julia embryos (average length  $1.64 \pm 0.13$  mm) was determined in the standard assay medium and with the addition of three uncoupling agents as described in Materials and Methods

Inhibitor treatment	Sucrose accumulation rate nmol h <sup>-1</sup> mg embryo d. wt <sup>-1</sup>	Inhibition (%)
—	325	—
Sodium arsenate (1 mM)	328	0
Sodium azide (1 mM)	152	53
2,4-DNP (0.01 mM)	187	43
2,4-DNP (0.10 mM)	161	51
2,4-DNP (0.50 mM)	199	38

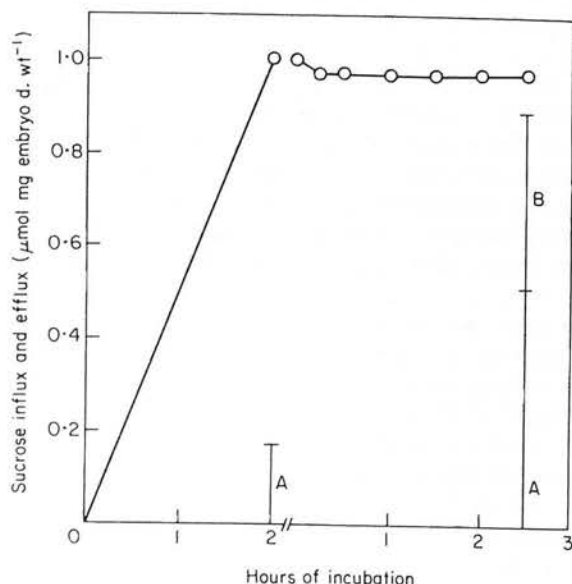


FIG. 5. Sucrose influx and efflux rates in immature barley embryos *in vitro*. Immature Midas embryos (average length  $2.27 \pm 0.13$  mm) were incubated in 5 mM phosphate buffer pH 5.0 with 100 mM [U- $^{14}$ C]sucrose at 25 °C for 2 h. The embryos were then washed and incubated in buffer alone for a further 2.5 h and the efflux of sucrose accumulated by the embryos in the first incubation was followed. At the end of each incubation, the accumulation of sucrose in the embryos and its distribution between 80 per cent ethanol insoluble (A) and soluble (B) fractions was determined.

The [ $^{14}$ C]sucrose pool size after 2 h assimilation has been calculated to be approximately 200 mM, making the assumption that the sucrose was evenly distributed through the cell sap. The results clearly demonstrate the stability of the sucrose pool in immature embryos, and the size of the concentration gradient against which sucrose accumulation must operate.

#### *The specificity of sucrose accumulation*

A characteristic of carrier-mediated transport is its substrate specificity. The effect of the monosaccharides glucose and fructose on sucrose uptake by immature embryos was determined. The initial rate of sucrose uptake was measured in the standard assay medium and with the addition of increasing concentrations (10–100 mM) of the two monosaccharides. Neither sugar was found to have any influence on sucrose uptake, indicating the specific nature of sucrose active transport by immature embryos.

### DISCUSSION

The transport of carbohydrates assimilated in the photosynthesizing leaf to the storage or growing meristematic tissues of higher plants is essential to their growth, yet our knowledge of the transport mechanisms involved is severely limited when compared with that of bacterial or animal systems. However, there is increasing evidence that active transport is involved in many aspects of unicellular and multicellular plant life. For example, active transport has been implicated in phloem loading (Geiger, 1976; Giaquinta, 1977; Malek and Baker, 1977), secretion by nectar glands (Lüttge and Schnepf, 1976) and nutrient assimilation by green algae (Tanner, 1969). Owing to the relative simplicity of the unicellular system for such studies, our understanding of the energetics of membrane

transport in the plant kingdom has shown the greatest advances in the green alga *Chlorella vulgaris*, where a model for sugar transport involving a proton-motive force has been proposed (Tanner *et al.*, 1974).

The study of membrane transport in multicellular plant systems is thwarted by the difficulties of introducing the transport substrate without damaging the cells and thereby influencing the results. In this respect, the advantages of the immature barley embryo for the *in vitro* study of membrane transport in higher plants are undeniable. The growth of the embryo depends on the assimilation of nutrients from the endosperm, in which the epithelial cells of the scutellum play a central role. At the early stages of embryogenesis the endosperm is a soft, watery tissue and the embryo can be isolated intact for *in vitro* studies.

In this paper it has been shown that sucrose is accumulated by immature barley embryos *in vitro*. Although sucrose uptake increases with embryo development, a comparison of the specific activity of sucrose accumulation has revealed that the uptake processes are fully operative early in embryogenesis. Sucrose uptake at substrate concentrations of < 50 mM observe saturation kinetics, characteristic of facilitated transport. At higher sucrose concentrations diffusion makes a significant contribution to sucrose influx. By extrapolating from the linear portion of a Lineweaver and Burke plot of sucrose uptake, it can be calculated that facilitated transport accounts for around 60 per cent of uptake when the sucrose concentration is 100 mM.

The sensitivity of sucrose uptake to uncoupling agents indicates that the transport process is active. Since the inhibitor experiments were performed at a sucrose concentration of 100 mM, a 53 per cent inhibition of uptake obtained with 1 mM sodium azide may lead us to conclude that the mediated portion of sucrose transport is a fully active process. Sodium arsenate, an uncoupler of substrate level phosphorylation, had no effect on sucrose uptake, indicating that its transport may not be directly coupled to glycolytic energy. If the sucrose concentration in the developing barley endosperm is similar to that in wheat, namely 58 mM (Jenner, 1976), then active transport of sucrose by growing embryos would be expected to play an important role in their assimilation of carbohydrates.

When sucrose is taken up by immature embryos *in vitro*, the majority accumulates in a pool of free sucrose and only some 20 per cent is utilized for macromolecular synthesis. The stability of the free sucrose pool, irrespective of the external sucrose concentration, and the specificity of the transport carrier for sucrose lend further support to the theory that sucrose uptake by barley embryos is mediated by active transport. During prolonged storage the gradual depletion of the sucrose pool of isolated embryos by respiratory and biosynthetic metabolism leads to an enhancement in the rate of sucrose transport. A regulatory role of the intracellular sucrose pool with respect to the accumulation of sucrose has similarly been shown in castor bean cotyledons (Komor, 1977).

The advantages of the embryo for studying membrane transport in higher plants has been widely appreciated, in particular in germinating castor beans. Here the lipid reserves in the endosperm are converted to carbohydrate and the embryo accumulates sucrose via the cotyledons, which lie in immediate contact with the endosperm (Kriedemann and Beevers, 1967*a, b*). As with barley embryos their accumulation of sucrose displays biphasic kinetics. This comprises a specific and high-affinity-active transport system ( $K_m = 25$  mM sucrose) at low substrate concentrations and a physical diffusion process at high substrate concentrations (Komor, 1977). The  $V_{max}$  of sucrose active transport in castor bean ( $150 \mu\text{mol h}^{-1} \text{g f. wt}^{-1}$ ) is significantly higher than that of barley embryos ( $39 \mu\text{mol h}^{-1} \text{g f. wt}^{-1}$ , reported here) or maize scutellum slices ( $45 \mu\text{mol h}^{-1} \text{g f. wt}^{-1}$ , Humphreys, 1973). It has been proposed that an electrogenic  $\text{H}^+$  pump, dependent on ATP hydrolysis, may drive sucrose transport in castor bean cotyledons (Hutchings, 1978*a, b*), whereby a carrier co-transporting  $\text{H}^+$  and a neutral sugar, moves

in response to the electrochemical gradient. The inhibition of sucrose transport in barley embryos by 2,4-DNP and azide may stem from the increased proton conductance of the membranes and associated loss of electrochemical potential caused by such uncouplers.

We may conclude that the demonstration of an active sucrose transport system in developing barley embryos is compatible with their need to compete effectively with the starch-synthesizing enzymes of the endosperm for available sucrose.

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# Morphology and Ultrastructure of Immature Cereal Grains in Relation to Transport

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## ABSTRACT

The surfaces of the pericarps of wheat and barley grains, 15-30 days after anthesis, were examined. Stomata were found in the pericarp epidermis on the ventral side at the apical end in one variety of wheat and four varieties of barley. Layers which stained red with Sudan IV were observed on the pericarp epidermis and on either side of the testa in immature barley grains. The ultrastructure of these layers was investigated. It was concluded that the cuticular layer inside the testa is derived from the nucellus. The significance of these cuticular layers in relation to the supply of carbon dioxide to the photosynthesizing cells of the pericarp is discussed. The movement of photosynthate and oxygen produced in the pericarp is also considered.

Key words: *Hordeum vulgare* L., *Triticum aestivum* L., barley, wheat, cereal grain, cuticle, stomata, pericarp.

## INTRODUCTION

Several workers (Carr and Wardlaw, 1965; Kriedemann, 1966; Evans and Rawson, 1970; Duffus and Rosie, 1973; Radley, 1976) have confirmed the photosynthetic capacity of developing wheat and barley grains. (The term 'grain' in this case refers to spikelets from which glumes and paleae have been removed.) However, there is some doubt as to the source of the carbon dioxide fixed by the green pericarp cells of these grains. Carr and Wardlaw (1965) considered that, although wheat grains could fix externally supplied carbon dioxide, they would be unlikely to do so when enclosed in glumes and paleae. Evans and Rawson (1970) reported that in the wheat varieties Sonora and Gabo no net photosynthesis by grains in air took place at any stage in development, whereas in the wheat variety Pitic net photosynthesis in grains occurred until 9 days after anthesis. Kriedemann (1966) failed to find any stomata in the pericarp of wheat (cv. Sabre) and suggested that the carbon dioxide fixed by immature wheat grains was derived from grain respiration. Radley (1976) found that when she removed the outer pericarp, fixation of atmospheric  $^{14}\text{CO}_2$  by immature wheat grains increased by about ten-fold. She therefore concluded that the outer layer of pericarp restricted the entry of carbon dioxide to the grain.

The presence of a cuticular layer on the outside of the immature wheat grain was reported by Percival (1921) but little attention has been paid to its properties. Bradbury, MacMasters and Cull (1956) found stomata in the epidermis of the wheat pericarp (cv. Pawnee) but there have been no reports of stomata in the pericarp of barley.

The present work was undertaken to investigate those anatomical features which could affect the supply of carbon dioxide to the photosynthesizing layers of the pericarp.



Some consideration is given to the routes by which photosynthate and oxygen may leave the pericarp.

# MATERIALS AND METHODS

Barley (*Hordeum vulgare* L. var. *distichum* cv. Midas and Julia and *Hordeum vulgare* L. var. *hexastichum* cv. Senta, Scottish Bere and Albino lemma) and wheat (*Triticum aestivum* L. cv. Maris Dove) were grown under greenhouse conditions in daylight supplemented with mercury vapour lamps to give a 20 h day. Barley grains were taken from the middle of ears and their age was estimated subjectively in 'days after anthesis' as described by Williams and Duffus (1977). In the present work, grains aged between 15 and 30 days after anthesis were used since this is the period during which the chlorophyll content of the pericarp reaches its maximum (Duffus and Rosie, 1973).

For transmission electron microscopy transverse slices 1 mm thick were cut from the middle of the grain under a fixative containing 2.5 per cent glutaraldehyde in 0.025 M phosphate buffer pH 7.2. The tissue slices were kept in this fixative at 4 °C for 20 h, post-fixed at room temperature in 2 per cent (w/v) osmium tetroxide for 2 h, dehydrated in an acetone series and embedded in an epon/araldite resin. Thin sections, cut on an LKB ultramicrotome, were stained in uranyl acetate and lead citrate and examined in an AEI EM802 electron microscope at 60 kV. The tissues illustrated in Plate 1 B and H were taken from the upper and lower parts of a grain respectively and were embedded in Spurr's resin (Spurr, 1969).

For scanning electron microscopy, whole grains were fixed in glutaraldehyde, post-fixed in osmium tetroxide and dehydrated in an acetone series as for transmission electron microscopy. After critical point drying the grains were coated with gold and examined in a Cambridge Stereoscan electron microscope.

The distribution of stomata was further investigated using polystyrene replicas of the outer epidermis of the pericarp (Quarrie and Jones, 1977).

For light microscopy, sections cut from blocks prepared for electron microscopy were stained in toluidine blue. Free-hand sections of fresh grains were stained with Sudan IV C.I. No. 26105 to locate lipids (Jensen, 1962).

# OBSERVATIONS

## General morphology

In barley 15–30 days after anthesis, the grain is an ovoid structure, rounded on the dorsal side and having a furrow or 'crease' on the ventral side. The surface of the grain is glabrous except at the stigmatic or 'brush' end where there is a tuft of hairs.

In cross-sections of the grain different tissues can be distinguished (Plate 1 A, B). The outer part of the pericarp is a 'transparent layer' of non-chlorenchymatous cells bounded by an epidermis. Inside this are two to three layers of cross cells, the chlorophyll-containing cells of the pericarp. The cross cells in the two inner layers are tightly packed and elongated with their long axes perpendicular to the long axis of the grain, but cells in the third layer are less regular in shape, thus increasing the volume of intercellular space. At this stage of development the inner epidermis of the pericarp consists of a small number of widely spaced tube cells at the dorsal side of the grain. Inside the pericarp are the testa, consisting of two layers of cells, the nucellus, consisting of a single layer of cells, the aleurone and the endosperm. The vascular bundle of the pericarp is in the furrow or 'crease' on the ventral side of the grain.

## Pericarp outer epidermis

The surfaces of 15- to 30-day wheat and barley grains were examined using scanning electron microscopy. In general, the cells of the outer epidermis are elongated in the

long axis of the grain and about three times as long as broad, but the epidermal cells in the crease are longer and narrower and those at the base of the grain on the dorsal side are almost isodiametric in outline (Plate 1C, D). Stomata were observed on wheat grains towards the brush end on the flanks of the crease (Plate 1E), but a search of the same region on barley grains failed to reveal any. However, when polystyrene replicas of the surfaces of 20- to 30-day grains of several varieties of barley (Senta, Midas, Julia and Albino lemma) were examined, up to ten stomata were observed on the ventral side of each grain among, and immediately below, the hairs at the base of the brush (Plate 1F). Between 15 and 30 days after anthesis in barley and wheat when the pericarp is green, the part of the grain in which stomata were found is only loosely covered by flowering glumes. The top of the barley grain appears above the palea for a short time during this phase of development and is the last part of the pericarp to fuse with the palea as the grain matures.

Scanning electron microscopy of a broken cell of barley pericarp epidermis indicated that a cuticular layer was present on the outer surface (Plate 1G). This was confirmed by light microscopy of free-hand sections of fresh 20- to 30-day grains of wheat and barley. In these, a thin continuous layer on the outside of the epidermis stained red with Sudan IV demonstrating the presence of a lipid layer. Transmission electron microscopy showed that the epidermal cells were bounded by an electron dense layer (Plate 1H). At higher magnification this was seen to have a 'bubbly' boundary on the inside and electron dense material interspersed with electron-transparent lamellae to the outside (Plate 2A).

#### *Inner layers of the pericarp*

During the phase of grain development when the cross cells are green and the endosperm is expanding, the contents of the non-chlorenchymatous layers immediately outside the cross cells degenerate, leaving a layer of crushed cell walls (Plate 1A). Neither in these crushed cell walls, nor in the walls of the cross cells and tube cells, was any cuticular material detected. Numerous plasmodesmata were observed between cross cells of the same layer (Plate 1B), but no plasmodesmata were found between the pericarp and the testa.

#### *Testa*

Plate 1A and B show that the testa in barley is made up of two layers of cells having different characteristics, those next to the green layer being smaller and having denser cytoplasm and fewer vacuoles than those next to the nucellus. On the outside of the outer layer of cells there is a cuticular layer which is continuous right round the grain except for the region immediately inside the vascular tissue. In sections of fresh material this layer stains red with Sudan IV in the same way as the cuticular layer on the outer epidermis of the grain, but is considerably thicker than the latter. At high magnifications in transmission electron microscopy the cuticular layer outside the testa is seen to be composed of a wide, lightly-stained region through which is spread a network of electron-dense material. This is bounded by a thin homogeneous layer outside which is a fine electron-dense lamella (Plate 2B).

On the inside of the testa is a thin layer which stains red with Sudan IV. It too is continuous round the grain except at the crease. Transmission electron microscopy of thin sections shows this to be similar in structure to the cuticular layer outside the testa but more electron dense. It consists of a region which is infiltrated with a network of electron-dense material and has a 'bubbly' boundary with the thick wall of the nucellus. Outside the reticulate zone is a narrow band of less electron-dense material separated from the inner wall of the testa by a thin electron-dense lamella (Plate 2C). If, during grain growth or specimen preparation, layers of cells become separated, this cuticular

layer remains attached to the testa (Plate 2D). The nucellar cell in Plate 2D contains a large concentration of rough endoplasmic reticulum near the wall. Outside the cell membrane there is an accumulation of electron-dense material similar to that in the cuticular layer.

## DISCUSSION

This investigation has shown that the cuticular layers on either side of the testa of barley are similar in structure to each other and to the outer cuticular membrane of the testa of the developing wheat grain described by Morrison (1975). Morrison did not find any reticulate component in the inner cuticular membrane and so concluded that the two cuticular membranes were different in both structure and chemical composition. However, preliminary observations in this laboratory on both wheat and oats (*Avena sativa* L. cv. Sentor) indicate that the cuticular layer on the inside of the testa does have a reticulate component and differs from the cuticular layer on the outside only in width and intensity of staining with heavy metals.

Contrary to the views of Pugh, Johann and Dickson (1932) and Krauss (1933), Morrison (1975) concluded from his developmental studies that the inner cuticle in the wheat grain is derived from the nucellar epidermis. In the present work the same conclusion has been reached, based not on developmental studies but on fine structure. The reticulate component of the cuticular layer inside the testa is situated next to the wall of the nucellus. In the cuticular layer outside the testa and the cuticular layer on the epidermis of *Avena* coleoptile (O'Brien, 1967) the reticulate component is situated next to the wall from which it originated. The presence of much rough endoplasmic reticulum and of cuticular-type material at the outer edge of nucellar cells (Plate 2D) is additional evidence that the inner cuticular layer originates in the nucellus. The fact that the inner cuticular layer adheres more strongly to the outer wall of the testa than to the wall of the nucellar cells may have led other workers to assume that it originated in the testa.

The existence of a cuticle on the outer epidermis of the barley pericarp and the absence of stomata from most of its surface would appear to support the suggestion of Radley (1976) that the outer pericarp limits the supply of atmospheric carbon dioxide to the photosynthesizing cells. However, it must be remembered that significant levels of dark respiration for intact grains have been recorded (Carr and Wardlaw, 1965; Evans and Rawson, 1970) and much of the carbon dioxide measured in these experiments had presumably diffused out through three cuticular layers. Data on the solubility of gases in fats and oils (Vibrans, 1935; Schaffer and Haller, 1943) supports the view expressed by Sorokin (1966) that lipids, the main chemical components of cuticular material, are highly permeable to carbon dioxide and virtually impermeable to oxygen, nitrogen and some other gases. Thus it may be that in Radley's experiments, removal of the outer layer of pericarp increased the rate of uptake of  $^{14}\text{CO}_2$  from the atmosphere, not perhaps by increasing the accessibility of carbon dioxide to the inner pericarp but because, in the intact grain, oxygen produced in photosynthesis was unable to escape and was therefore inhibiting carbon dioxide fixation. Two enzymes responsible for carbon dioxide fixation are present in barley pericarp tissue, ribulose biphosphate carboxylase (E.C. 4.1.1.39) and phosphoenolpyruvate carboxylase (E.C. 4.1.1.31) (Duffus and Rosie, 1973). The former is inhibited by oxygen whereas the latter is not. This means that in intact grains where there would be a build-up of oxygen in the light, carbon dioxide would be fixed by phosphoenolpyruvate carboxylase. Removal of the outer layer of the pericarp would then allow both enzymes to function and the rate of carbon dioxide fixation would increase. The low permeability of cuticular material to oxygen may also explain the fact that in barley, the removal of the outer layer of pericarp resulted in an increased oxygen uptake in the dark (Nutbeam and Duffus, 1978).

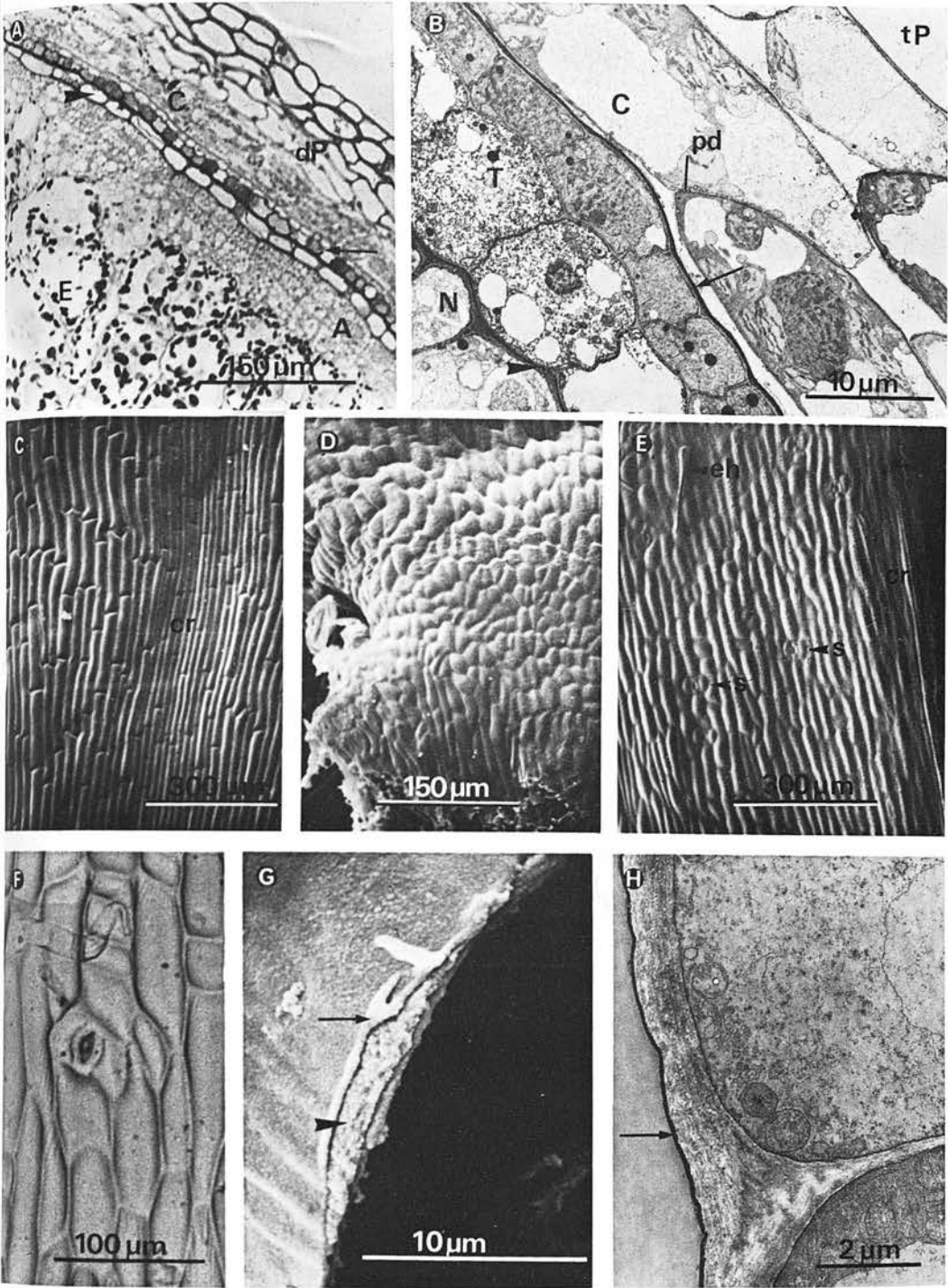


PLATE 1



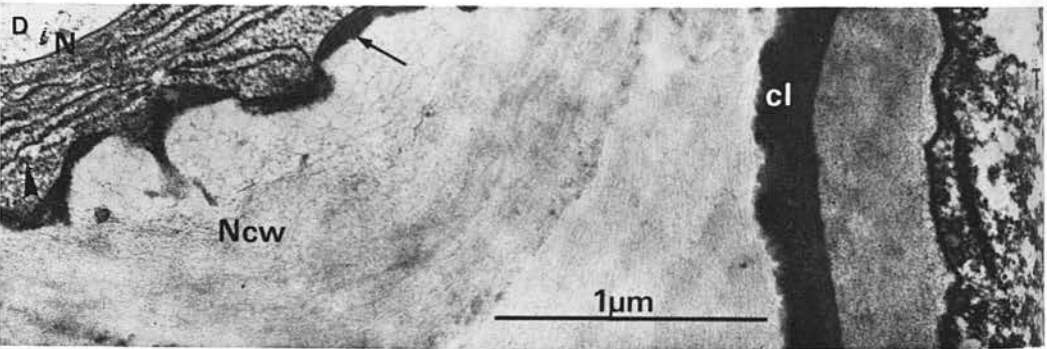
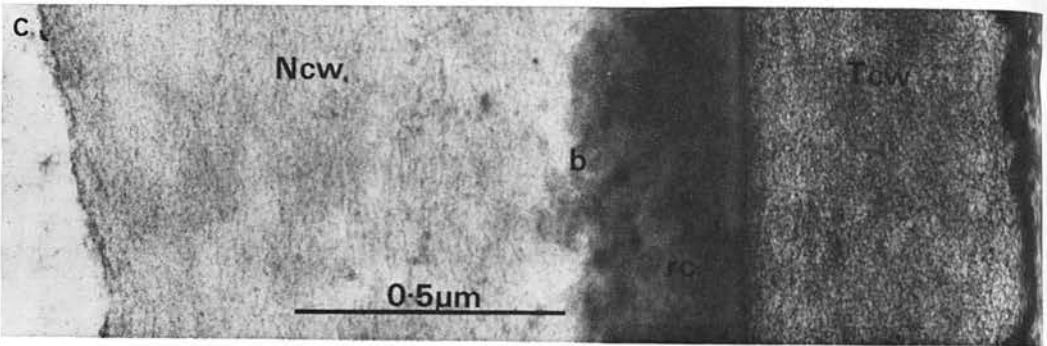
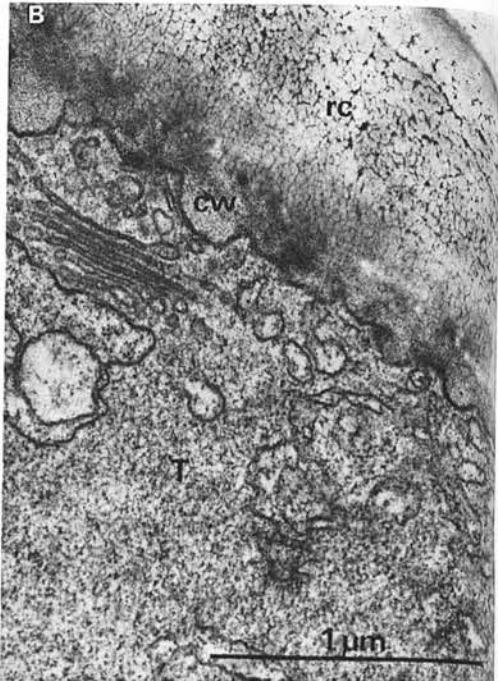
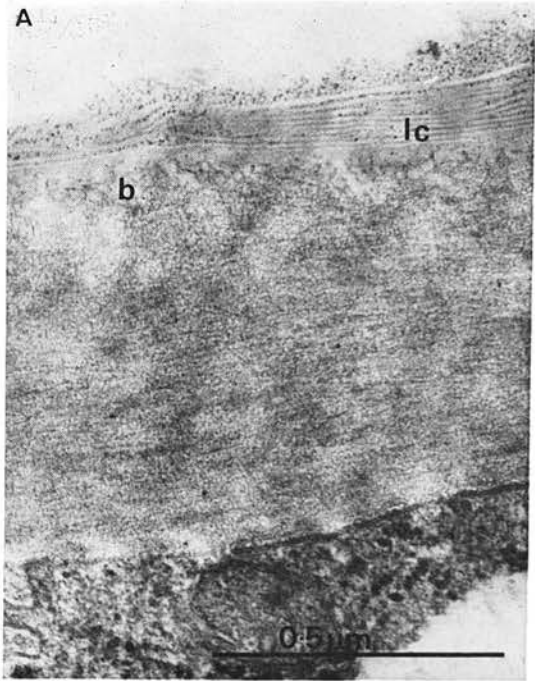


PLATE 2

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The stomata on the pericarps of barley and wheat are very few in number but, being situated at the brush end of the grain, they are not tightly covered by paleae during the phase of development when the pericarp is photosynthesizing. In addition, the anatomy of the developing grains is such that the volume of air space in and around the photosynthetic tissue is greater at the brush end than in other parts of the grain (Alexandrov, 1943; Bradbury *et al.*, 1956). It is therefore likely that the stomata of the grain do function in gas exchange.

The route by which photosynthate from the pericarp reaches the endosperm has yet to be established. Plasmodesmata between cross cells reported by Morrison (1976) in wheat pericarp have also been observed in barley (Plate 1B). Thus it is probable that there is symplastic movement of metabolites round the grain to the vascular bundle and thence to the endosperm. However, since preliminary observations show that the 'maximum cells distant count', i.e. the maximum number of cells separating any chlorenchymatous cell from its nearest vascular bundle (Hattersley and Watson, 1975), is three in barley leaves but about 70 in barley pericarps, this route is long and inefficient. One possibility is that apoplastic transport of metabolites takes place through the cuticular layers of the testa and nucellus. Movement of sugars through cuticular layers has been reported by Gunning and Steer (1975). It is also possible that metabolites reach the vascular bundle of the pericarp by apoplastic transport along the cell wall material which remains after the breakdown of the inner cells of the transparent layer. Transport in this region would not necessarily be limited to metabolites but could also include oxygen and carbon dioxide.

From these considerations it appears that carbon dioxide can reach the green layer of the pericarp from the atmosphere, by diffusion through stomata and the surface of the pericarp epidermis, and from the endosperm, by diffusion through the cuticular layers of the testa and nucellus. Since oxygen is scarcely soluble in cuticular material the main escape route for oxygen produced in the pericarp is through stomata. These are very few in number and so oxygen probably accumulates outside the cross cells, from where some of it may move round the grain and enter the endosperm via the vascular bundle. Experimental work will be needed in order to confirm this and to establish the route by which metabolites from the pericarp reach the endosperm.

#### ACKNOWLEDGEMENTS

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## EXPLANATION OF PLATES

## PLATE 1

- A. Transverse section of the dorsal side of a grain of 25 day barley (Julia) showing the relative positions of nucellus (arrowhead), testa (arrow), endosperm (E), aleurone (A), cross cells (C), degenerating cells of transparent pericarp (dP).  $\times 150$ .
- B. Transverse section through the dorsal side of the upper part of a 20 day barley (Julia) grain. The testa (T) is separated from the pericarp by the outer cuticular layer (arrow) and from the nucellus (N) by the inner cuticular layer (arrowhead). Transparent pericarp (tP) lies outside cross cells (C) which are interconnected by numerous plasmodesmata (pd).  $\times 1900$ .
- C. Scanning electron micrograph of part of the ventral surface, including the crease (cr), at the equator of a 25 day grain of barley (Julia).  $\times 65$ .
- D. Scanning electron micrograph of the ventral surface at the base of a 25 day grain of barley (Julia).  $\times 135$ .
- E. Scanning electron micrograph of stomata (s) near epidermal hairs (eh) on the flank of the crease (cr) at the brush end of an immature wheat grain.  $\times 70$ .
- F. Polystyrene replica of a stoma in the pericarp epidermis of barley (Senta).  $\times 190$ .
- G. Scanning electron micrograph of a broken epidermal cell of a 25 day barley (Julia) grain showing the cell wall (arrowhead) and a cuticular layer (arrow), (not post-fixed in osmium tetroxide).  $\times 3200$ .
- H. Transverse section of the cuticular layer (arrow) on an outer epidermal cell wall near the base on the ventral side of a 20 day grain of barley (Julia).  $\times 7700$ .

## PLATE 2

- A. Transverse section of an outer epidermal cell wall of a 30 day grain of barley (Scottish Pere) showing the 'bubbly' boundary (b) between the lamellate cuticular layer (lc) and the cell wall.  $\times 75000$ .
- B. Transverse section showing the reticulate component (rc) of the cuticular layer on the outer wall (cw) of the testa (T) in a 25 day grain of barley (Julia).  $\times 37000$ .
- C. Transverse section of the boundary between the testa cell wall (Tcw) and nucellus on the dorsal side of a 30 day grain of barley (Scottish Bere) showing the 'bubbly' boundary (b) between the cell wall of the nucellus (Ncw) and the reticulate component of the cuticular layer (rc).  $\times 70000$ .
- D. Transverse section of the boundary between testa (T) and nucellus (N) on the dorsal side of an 18 day grain of barley (Julia). There is rough endoplasmic reticulum (arrowhead) in the nucellar cell and cuticular-type material (arrow) lying outside the plasmalemma. The cuticular layer (cl) is not attached to the cell wall of the nucellus (Ncw).  $\times 35000$ .

## Starch synthesis and grain growth

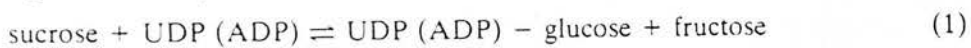
Carol M. Duffus

### The source of grain carbohydrate

The dry matter entering the developing barley grain is derived mainly from photosynthesis occurring after ear emergence, the ear itself contributing a major part of the carbon dioxide fixed (Frey Wyssling & Buttrose, 1959). The tissues of the ears of wheat, barley, oats and rye which may supply carbohydrate for grain filling include the awns, glumes, lemmas and paleae. Furthermore the grain itself may account for as much as 40% of gross photosynthesis, derived from carbon dioxide fixation in the chlorophyll-containing layers of the pericarp (Nutbeam & Duffus, 1976). Sucrose is generally considered to be the major carbohydrate reaching the grain from the flag leaf. The exact composition of the sugars entering the grain from this source is not known, however, since the phloem is a notoriously difficult tissue to analyse. The end-product of pericarp photosynthesis is sucrose (Nutbeam & Duffus, 1976) and this has been shown to be a precursor of endosperm starch granules (A. R. Nutbeam: personal communication). One conclusion from these observations is that much of the sucrose and other metabolites reaching the grain may be derived, not from such traditional structures as leaves, but from rather less orthodox sources about which relatively little is known.

### Sucrose utilization

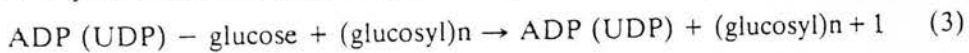
In rice (Perez et al., 1975), barley (Baxter & Duffus, 1973) and sweet corn endosperms (de Fekete & Cardini, 1964) it is considered that the sucrose entering the grain is mainly metabolized by UDP-independent sucrose synthase:



In the early stages of embryo (Duffus & Rosie, 1975) and maize endosperm (Tsai & Nelson, 1969) development however, sucrose may initially be cleaved by invertase:



Thus the products of sucrose metabolism are mainly UDP-glucose with some ADP-glucose and free glucose and fructose. The preferred nucleotide sugar donor for starch synthesis, on the other hand, is thought to be ADP-glucose:



It may be that UDP-glucose is first converted to glucose-1-phosphate via UDP-glucose pyrophosphorylase and thence to ADP-glucose by a reversal of ADP-

total activity is granule bound (Ozbun et al., 1973). However, maximum activity *in vitro* may not be obtained when the enzyme is complexed intimately with the starch granule. Of course this may also be the case *in vivo*.

The presence of starch granules of different sizes and compositions (q.v.) suggests that there may be several amyloplast bound synthases. Certainly the granule bound synthases of rice (Perez et al., 1975) and barley (Baxter & Duffus, 1971) can use both UDP and ADP. This may be accounted for by the finding (Williams & Duffus, 1977) that the nucleotide specificity of the starch synthase associated with the small amyloplasts differs from that of the large. Furthermore the nucleotide specificity for the starch synthase of each population varies throughout grain development.

The biosynthesis of amylopectin has been little investigated in developing cereal grains, mainly because of difficulties in assaying the enzyme in the presence of interfering amylases (q.v.).

### *The starch granule*

The starch content of cereal grains increases steadily throughout development, accounting for around 65–70 per cent of grain dry weight at maturity. In general it seems that the amount of amylose relative to the amount of starch also increases throughout grain development. It seems also that, in a number of cereals, the largest granules have a significantly higher amylose content than the smallest. It would appear then, that the changes in composition of the starch may be due to changes in the relative numbers of starch granules of different sizes as well as to changes in the individual amylose contents of all granules (Duffus & Jennings, 1978).

### **Mutant endosperms**

A range of mutant genotypes affect grain polysaccharides. These have been used by biochemists to investigate mechanisms of starch synthesis but with comparatively little success. In maize the mutant genotypes include *sugary*, *waxy*, *amylose extender* (high amylose) and in barley, high amylose. Many, or most, of these have odd shaped granules. For example high amylose barley has smaller starch granules than the normal varieties. It was thought at one time that these smaller granules would be more easily degraded than the larger granules during malting, but in fact the mutant proved more resistant to its own enzyme system than the normal varieties (Ellis, 1976). The properties of starch granules which render them susceptible to enzyme attack are not understood. They do not appear to be related directly to amylose content, density, gelatinization temperature, or to the ability of the granule to absorb cold water. Further, the work with mutant endosperms has made it clear that the nature of the genetic and biochemical factors which control the size, shape and composition of starch granules are largely unknown.

### **Carbohydrate degradation during grain development**

That a considerable amount of carbohydrate is lost during the day by ear respiration has been shown by Thorne (Thorne, 1965). More specifically this has been

shown to result at least in part from catabolic processes active in starch hydrolysis and degradation during grain development. Obviously intermediates and energy derived from these are required for the great variety of synthetic processes carried out by endosperm cells. Both glycolytic (Duffus & Rosie, 1977) and mitochondrial (Duffus, 1970) enzyme activities are present, together with a number of starch degrading enzymes such as phosphorylase,  $\alpha$ - and  $\beta$ -amylases and debranching enzyme (Duffus & Rosie, 1973).

It may be that there is a relationship between the activity of such degradative enzymes and the growth rate and/or final yield of grain. No such relationship was found between grain growth rates and  $\alpha$ -amylase activity (Riggs & Gothard, 1976). However, activity of this enzyme is low and a relationship might more usefully be sought between growth rate, yield and total grain hydrolytic activity. The relative activities of a number of enzymes present in immature barley endosperm, involved in carbohydrate degradation, are shown in Table 1.

In addition to supplying keto-acids and energy it is likely that starch hydrolysis yields the primers required to maintain high rates of starch synthesis.

Little is known of the extent of the losses due to internal carbohydrate degradation. That there is aerobic metabolism is inferred from the presence of active (Duffus, 1970) and visible (Williams & Duffus, 1978) mitochondria, and it can be assumed that if keto-acids are required for amino acid synthesis then a substantial amount of carbon dioxide may be evolved as a consequence of their production.

### **The role of the chlorophyll-containing layer of the pericarp**

During most of the grain filling period the pericarp is a bright emerald green tissue surrounded by a transparent outer layer. It is active in carbon dioxide fixation and has a number of the biochemical characteristics of  $C_4$  photosynthesis (Nutbeam & Duffus, 1976; Wirth et al., 1977). Thus, the preferred enzyme for carbon dioxide fixation is PEP carboxylase rather than RBP carboxylase. Further, PEP synthetase, the enzyme responsible for regenerating the carbon dioxide acceptor in the  $C_4$  pathway, is present in both barley and wheat pericarps (Duffus & Rosie, 1973; Wirth et al., 1977). There is some evidence (Nutbeam, 1978) to suggest that the photosynthetic activity of the pericarps is directly related to yield. For example, the low yielding barley variety Scottish Bere has low PEP carboxylase activity compared to the variety Julia. The mutant barley *Albino lemma* with an albino pericarp similarly has low carboxylase activity and low yield.

Much of the carbon dioxide fixed doubtless is derived from endosperm respiration but some may also come directly from the atmosphere since immature barley and wheat pericarps do have small numbers of stomata (M.P. Cochrane, personal communication). The endosperm mitochondria depend on adequate supplies of oxygen, and, since there are barriers to oxygen efflux from the green layer of pericarp to the atmosphere (Nutbeam & Duffus, 1979), some may remain inside. It may be that this oxygen, by its presence or absence, may regulate endosperm respiration and hence the biosynthesis of endosperm storage material.

## Conclusion

The biosynthesis of storage material in cereal grains takes place against a background of active catabolic processes, with losses to some extent offset by refixation of respired and atmospheric carbon dioxide in the pericarp.

## Implications for plant breeding

Starch synthesis is limited, probably not by sucrose supply but by the low ADPG pyrophosphorylase activity (Table 1). Its composition, on the other hand, is related to a number of factors which are not fully understood. Since the mechanisms involved in starch biosynthesis are the subject of much speculation, little is to be gained at the present time by breeding for varieties with high ADPG pyrophosphorylase activity or even small/large starch granules.

More worthwhile is to look for varieties with high rates of pericarp carbon dioxide fixation. Simple measures of this may be chlorophyll content or rates of light-dependent oxygen evolution.

Hydrolytic activity in the endosperm may well be beneficial, since its products are used in amino acid synthesis. Thus hydrolytic activity may be correlated with high grain protein content, although at the same time yield may be reduced due to depletion of carbohydrate reserves.

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## Hexose Transport in Isolated Immature Barley Embryos

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### ABSTRACT

The pathways of monosaccharide assimilation in developing barley embryos have been investigated in an *in vitro* assay system, and the results are discussed in relation to the primary carbohydrate nutrients of the developing and germinating embryo. Glucose and fructose are both rapidly taken up by immature embryos, and at a substrate concentration of 100 mM their assimilation rate exceeds by threefold that of sucrose. The kinetics of hexose uptake are complex, reflecting a combination of passive diffusion and metabolically mediated uptake mechanisms. The hexose transport energetics were further studied with the glucose analogue 3-*O*-methyl-D-glucose, shown to be a non-metabolite in the barley embryo. The kinetics of 3-*O*-methyl-D-glucose influx and efflux indicated that passive diffusion accounts for a major part of hexose assimilation in barley embryos. However, an active transport component was revealed by the sensitivity of 3-*O*-methyl-D-glucose uptake to uncoupling agents and the demonstration of competitive inhibition of uptake between glucose analogues. These observations supported the view that part of the observed energy-dependence of hexose uptake might be associated with a direct coupling of energy to transport. A high-affinity active group translocation mechanism for glucose assimilation could be important in embryo metabolism during the first hours of germination prior to hydrolysis of endosperm starch reserves. Analysis of the fate of assimilated hexoses showed that the rapid consumption of the hexose pool in the synthesis of sucrose must contribute to the influx of hexoses by the maintenance of a hexose concentration gradient between the embryo and its environment.

Key words: *Hordeum distichum* (L.) Lam., barley, embryo, hexose transport.

### INTRODUCTION

The developing barley embryo primarily depends on the photosynthetic activity of the flag leaf and the ear itself for its supply of carbohydrate nutrients (Archbold, 1942; Frey-Wyssling and Buttrose, 1959). Sucrose, the major translocation sugar, is transported by the phloem from the photosynthetic tissues to the endosperm cavity of the grain by a combination of active phloem-loading and mass flow (Jenner, 1976). In wheat ears at least, the flow of sucrose into the endosperm free-space is regulated at some point between the rachis and the phloem in the grain furrow, allowing the sucrose concentration to reach a maximum of 58 mM (Jenner and Rathjen, 1972). The growth rate of immature barley embryos cultured *in vitro* has indicated that their development *in vivo* may be restricted by the supply of nutrients (Cameron-Mills and Duffus, 1977). An active sucrose transport mechanism has been demonstrated in isolated embryos and it has been postulated that this may be a key factor in the ability of the embryo to compete with the starch-synthesizing enzymes of the endosperm for available sucrose (Cameron-Mills and Duffus, 1979).

During the main period of embryo growth, 20-40 days after anthesis (Duffus and Rosie, 1975), reducing sugars comprise only 2-3 per cent of the grain d. wt (Baxter and Duffus, 1973) and glucose may be barely detectable (Cerning and Guilbot, 1973). Although monosaccharides may only play a minor part in the nutrition of developing

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embryos, glucose released on hydrolysis of the starch reserves is a primary nutrient for germinating embryos.

This paper describes the mechanism of glucose and fructose uptake found to operate in developing barley embryos and discusses their potential role during embryogenesis and germination.

## MATERIALS AND METHODS

The two-row barley variety *Hordeum distichum* (L.) Lam. cv. Midas was grown in a glasshouse at approximately 20 °C under natural light, extending the daylength to 18 h with Osram 400 W sodium lamps. The first six tillers of any plant were labelled on the day of anthesis, as described by Merritt and Walker (1969). The ears were harvested approximately 16 days for the selection of mid-growth phase embryos, which measure 1.8–2.2 mm in length (Cameron-Mills and Duffus, 1979). Twelve grains, from the central portion of each ear, were dehusked and sterilized in 5 per cent sodium hypochlorite for 10 min and thoroughly rinsed in sterile distilled water. Since anthesis dating is subject to a 1- to 2-day error and the main embryo growth period is only 10 days, the final selection of barley ears was based on the size of the isolated embryos.

The relationship between embryo length and d. wt (semi-log plot) is linear over a large portion of embryogenesis (Cameron-Mills and Duffus, 1979). Since the embryo size in all the described experiments was monitored, the results could be expressed on a d. wt basis by interpolation from an embryo length/d. wt correlation plot (Cameron-Mills and Duffus, 1979), thereby avoiding the problems of weighing small numbers of embryos.

The excision of embryos and subsequent uptake assays were performed under sterile conditions. From each selected ear one embryo was placed in each sample tube and stored on ice in 5 mM phosphate pH 5.0 prior to assay. Unless otherwise specified, several embryos were then incubated in 1 ml of medium containing 5 mM phosphate buffer pH 5.0 and a 100 mM concentration of either U-<sup>14</sup>C-labelled sucrose, D-glucose, D-fructose or 50 mM 3-O-methyl D-[U-<sup>14</sup>C]glucose, all at a specific activity of 1 µCi per 100 µmol. The samples were placed in a shaking water-bath at 25 °C to determine their uptake of sugars. To terminate each assay, the embryos were collected by Millipore filtration and washed in non-radioactive medium. The 80 per cent aq. ethanol-soluble sugars were extracted at 70 °C with three successive volumes of solvent and the pooled extracts were dried down under vacuum. After re-solubilization in 80 per cent ethanol the samples were pipetted on to glass fibre discs which were dried and suspended in toluene-2,5-diphenyl oxazole-1,4-bis(5 phenyloxazol-2-yl) benzene scintillant for radioactivity measurements in a Beckman LS 3155T scintillation spectrophotometer. Radioactivity remaining in the embryo 80 per cent ethanol-insoluble fraction was determined with the aid of a Packard Tricarb Sample Oxidiser 306.

Chromatographic separation of sugars was performed on Whatman No. 1 paper using a descending system for 18 h in a solvent of ethyl acetate:pyridine:water (10:4:3, v/v/v). The 80 per cent ethanol extracts from 1 mg embryo dry weight were co-chromatographed with 20 µg each of a range of marker sugars. Mono-, di- and oligosaccharides were detected with a non-specific silver nitrate dip (Trevelyan, Proctor and Harrison, 1950). The monosaccharides were distinguished with an anisidine spray reagent (Pridham, 1950) and the phosphorylated sugars with an ammonium molybdate reagent (Hanes and Isherwood, 1949). Kodak materials were used for the autoradiography of the paper chromatograms.

## RESULTS

### *Monosaccharide assimilation by immature barley embryos in vitro*

Glucose and fructose uptake by immature embryos from the standard assay medium containing 100 mM [U-<sup>14</sup>C]hexose was measured. Both sugars were rapidly taken up into

the 80 per cent ethanol extract of monosaccharides. The initial rate of uptake was greater than that of fructose.

TABLE 1  
The rate of uptake of monosaccharides by immature barley embryos.

FIG. 2  
The rate of uptake of monosaccharides by immature barley embryos.

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The initial rate of uptake of monosaccharides by immature barley embryos. The rate of uptake of monosaccharides by immature barley embryos.

the 80 per cent ethanol-soluble fraction of the embryos (Fig. 1) and after 6 h there was little diminution in the rate of uptake. In order to assess accurately the comparative rates of mono- and disaccharide uptake by embryos, the initial rate of glucose, fructose and sucrose uptake was determined within a single group of embryos (Table 1). While the initial rates of glucose and fructose uptake were almost identical, they were threefold greater than that of sucrose.

TABLE 1. Comparative rates of sugar uptake by immature barley embryos *in vitro*. The rates of glucose, fructose and sucrose uptake by immature embryos (average length 1.90 mm) were determined in the standard assay medium over a 2 h incubation as described in Materials and Methods

Substrate (100 mM)	Sugar uptake rate	
	nmol per 2 h per 10 embryos	nmol per 2 h per mg embryos d. wt)
Glucose	970	970
Fructose	920	980
Sucrose	290	300

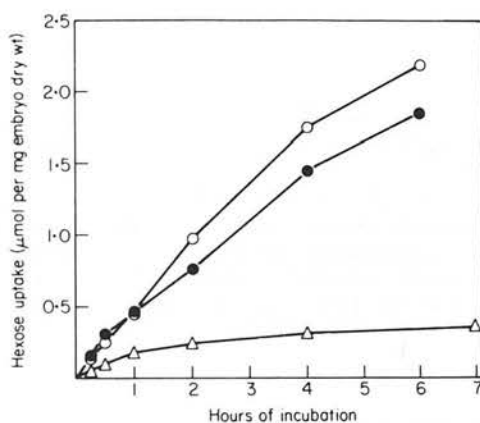


FIG. 1. Monosaccharide uptake by immature barley embryos *in vitro*. Isolated immature embryos (average length 1.85 mm, 2.00 mm and 1.9 mm respectively) were incubated in 5 mM phosphate buffer pH 5.0 containing 100 mM ( $U-^{14}C$ ) labelled glucose (○) or fructose (●) or 50 mM 3-O-methyl-D-( $U-^{14}C$ ) glucose (△), (specific activity 1  $\mu Ci$  per 100  $\mu mol$ ) at 25 °C in a shaking water bath. Samples of embryos (7) were harvested at intervals and the uptake of each sugar into the 80 per cent ethanol-soluble fraction determined.

#### Characterization of monosaccharide uptake

The initial rates of glucose and fructose uptake by immature embryos were determined at substrate concentrations ranging from 5 to 100 mM [Fig. 2(a)]. While the rate of hexose uptake increased with the substrate concentration, the kinetics did not precisely fit the saturation kinetics characteristic of carrier-mediated membrane transport. This, in addition to the high  $K_m$  of hexose uptake [glucose  $K_m$  100 mM and fructose  $K_m$  167 mM, Fig. 2(b)], indicated that a combination of passive diffusion and facilitated uptake might operate in hexose uptake by immature embryos.

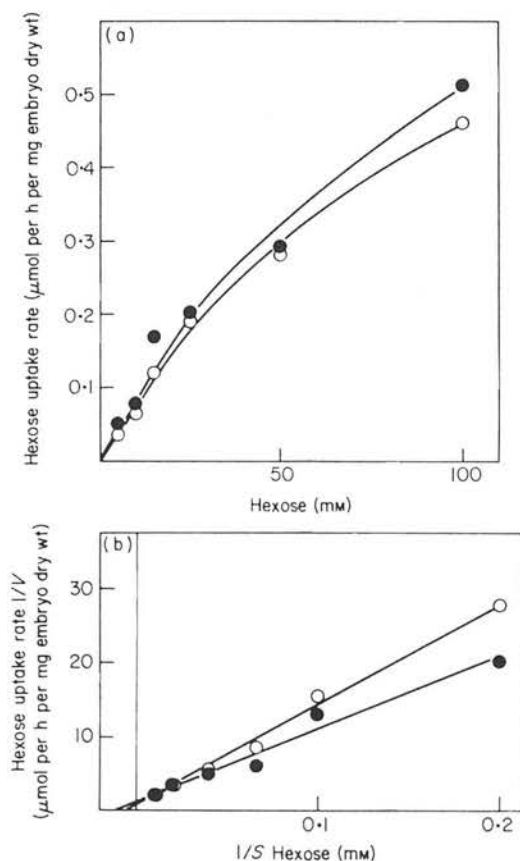


FIG. 2. The kinetics of glucose and fructose uptake by immature barley embryos *in vitro*. (a) The initial rate of glucose (○) and fructose (●) uptake by immature embryos (average length 1.82 mm and 1.76 mm respectively) was determined at a range of substrate concentrations in the standard assay medium as described in Fig. 1. (b). The sugar uptake data in (a) are represented in a double reciprocal plot.

#### Sensitivity of monosaccharide uptake to metabolic inhibitors

The demonstration of energy-dependence is a central feature in the identification of an active transport system. An important energy-coupling mechanism involves the interaction of components of the electron transfer chain with the transport carrier through one or more intermediates. The effect of three uncoupling agents on the initial rate of glucose uptake by immature embryos was investigated (Table 2). Although sodium arsenate (1 mM), an inhibitor of substrate level phosphorylation, had no effect on glucose uptake, two uncouplers of oxidative phosphorylation [sodium azide (1 mM) and 2,4-DNP (0.1 mM)] inhibited uptake by over 50 per cent. While glucose uptake was shown to be energy-dependent, the transport process itself was not necessarily the primary active step. A concentration gradient between the embryo and its environment sufficient to drive glucose influx could be maintained by the further metabolism and consequent depletion of the glucose pool.

#### Non-metabolizable glucose analogues for the study of monosaccharide uptake

3-O-Methyl-D-glucose (3-O-MG) has successfully been used in a number of plant systems as a non-metabolizable glucose analogue for the study of hexose transport.

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TABLE 2. The effect of metabolic inhibitors on *in vitro* glucose uptake by immature barley embryos. The initial rate of glucose uptake by immature embryos (average length 1.83 mm) was determined in the standard assay medium and with the addition of three uncoupling agents as described in Materials and Methods

Inhibitor treatment	Glucose uptake rate nmol per h per mg embryo d. wt	per cent inhibition
—	440	—
Sodium arsenate (1 mM)	470	0
Sodium azide (1 mM)	210	53
2,4-DNP (0.01 mM)	290	34
2,4-DNP (0.10 mM)	210	53
2,4-DNP (0.50 mM)	240	46

Since in some instances 3-*O*-MG uptake was found to be considerably slower than glucose (Komor and Tanner, 1971) the potential of this analogue in the barley embryo system was first investigated. When immature embryos were incubated with 50 mM 3-*O*-M[U-<sup>14</sup>C]G measurable amounts were taken up, although the initial rate of uptake was only one-third that of glucose (Fig. 1). The 3-*O*-MG pool approached saturation after 7 h and assuming free sugars to be evenly distributed through the cell sap the intracellular concentration could be calculated to be 67  $\mu$ M.

Before the potential of 3-*O*-MG for the study of hexose uptake could be exploited it was essential to prove that this analogue was a non-metabolite in barley embryos. The fate of 3-*O*-M[U-<sup>14</sup>C]G taken up into the 80 per cent ethanol-soluble fraction of embryos incubated with 3-*O*-M[U-<sup>14</sup>C]G for 7 h was thus investigated. The mono- and disaccharide constituents of the 80 per cent ethanol extracts were separated by paper chromatography and many of the pool sugars identified with the aid of detection agents and marker sugars (see Materials and Methods; Cameron-Mills and Duffus, 1979). After autoradiography of the chromatograms some 83 per cent of the radioactivity applied to the papers could be accounted for as 3-*O*-MG, while the remaining 17 per cent was associated with phosphorylated derivatives.

Measurement of the radioactivity in the 80 per cent ethanol-insoluble fraction of these embryos revealed that less than 1 per cent of the 3-*O*-MG taken up is incorporated into macro-molecular material. Bearing in mind the short incubation time of most of the projected experiments, in the context of these studies 3-*O*-MG was defined as a non-metabolite for barley embryos.

#### Characterization of 3-*O*-methyl-D-glucose uptake

The initial rate of 3-*O*-M[U-<sup>14</sup>C]G uptake by immature embryos at substrate concentrations between 5 and 100 M (Fig. 3). Within this concentration range there was little evidence of a saturation of 3-*O*-MG uptake capacity. A high  $K_m$  of 167 mM for 3-*O*-MG uptake, derived from a double reciprocal plot of the data (not shown), was also a feature of glucose and fructose uptake.

The kinetics of 3-*O*-MG uptake *in vitro* indicate that passive diffusion must account for a major part of monosaccharide assimilation in developing embryos.

#### Sensitivity of 3-*O*-methyl-D-glucose uptake to metabolic inhibitors

The effect of uncoupling agents on the uptake of 3-*O*-M[U-<sup>14</sup>C]G by immature embryos was investigated, and those uncouplers of oxidative rather than substrate level phosphorylation were found to have a marked inhibitory effect (Table 3). This inhibition

pattern was similar although smaller than that of glucose uptake (Table 2). These results indicate that while a part of energy-dependent hexose uptake in embryos may be attributable to the effects of secondary metabolism, a significant portion may involve a more direct coupling of energy and transport.

### Stability of the 3-O-methyl-D-glucose pool

An important attribute of active transport mechanisms is the accumulation of nutrients against a concentration gradient and their retention in a pool, irrespective of the external substrate concentration. The ability of immature embryos to retain accumulated hexoses against a concentration gradient was investigated with 3-O-MG.

Isolated embryos were pre-loaded with 3-O-M[U-<sup>14</sup>C]G by incubation in the standard assay medium with 100 mM-3-O-M[U-<sup>14</sup>C]G for 2 h. The embryos were then harvested and washed in 100 mM glucose in buffer solution and a sample of embryos removed to determine the amount of 3-O-MG accumulated. The remaining embryos were incubated in buffer alone, from which samples were removed after various intervals to determine the 3-O-MG efflux rate. After 3 h incubation the embryos were harvested and the amount of 3-O-MG in the 80 per cent ethanol-soluble and insoluble fractions determined (Fig. 4).

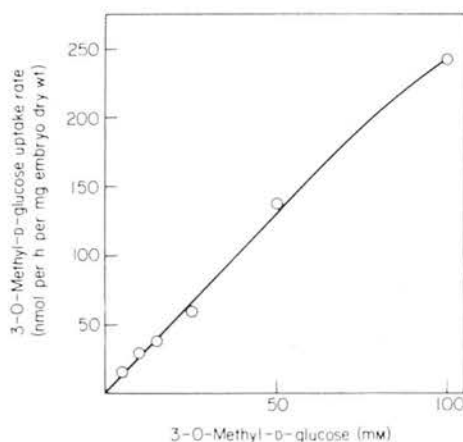


FIG. 3. The kinetics of 3-O-methyl-D-glucose uptake by immature barley embryos *in vitro*. The initial rate of 3-O-methyl-D-(U-<sup>14</sup>C) glucose uptake by immature embryos (average length 2.40 mm) was determined at a range of substrate concentrations in the standard assay medium as described in Fig. 1.

TABLE 3. The effect of metabolic inhibitors on the *in vitro* uptake of 3-O-methyl-D-glucose by immature barley embryos. The uptake of 3-O-methyl-D-glucose by immature embryos (average length 1.90 mm) was determined over 2 h in the standard assay medium and with the addition of four uncoupling agents as described in Materials and Methods

Inhibitor treatment	3-O-methyl-D-glucose uptake rate nmol per 2h per mg embryo d. wt	Per cent Inhibition
—	180	—
Sodium arsenate ( mM)	180	0
Sodium azide (1 mM)	120	33
2,4 DNP (0.1 mM)	110	38
FCCP (0.02 mM)	120	34
FCCP (0.05 mM)	140	24

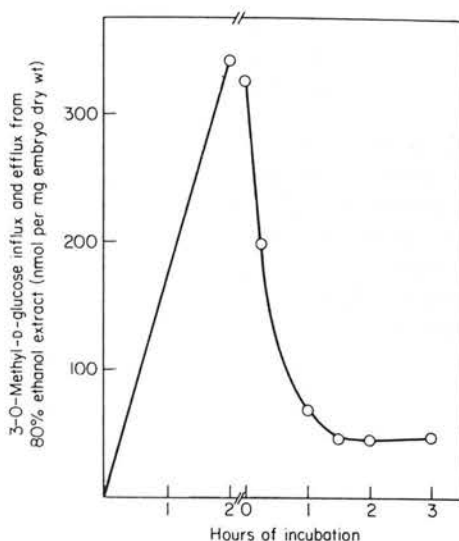


FIG. 4. 3-*O*-Methyl-D-glucose influx and efflux in immature barley embryos *in vitro*. Immature embryos (average length 2.15 mm) were incubated in 5 mM phosphate buffer pH 5.0 with 100 mM 3-*O*-methyl-D-(U- $^{14}$ C) glucose at 25 °C for 2 h. The embryos were then washed and incubated in buffer alone for a further 3 h, and the efflux of 3-*O*-methyl-D-glucose accumulated by the embryos in the first incubation was followed. At the end of each incubation the accumulation of 3-*O*-methyl-D-glucose in the embryos and its distribution between 80 per cent ethanol-soluble and insoluble fractions was determined.

During the first incubation 3-*O*-MG was accumulated rapidly, of which less than 0.1 per cent was incorporated into macro-molecular material. After 90 min of the second incubation in buffer, over 86 per cent of the 3-*O*-MG pool was lost by efflux. Of the residual 3-*O*-MG, 1.9 per cent was fixed in the 80 per cent ethanol-insoluble fraction and 12 per cent was retained in the pool, but none was consumed by respiratory metabolism. An intracellular 3-*O*-MG concentration at the end of the first and second incubations of 75 and 11  $\mu$ M respectively has been calculated.

The results clearly demonstrate that *in vitro*, immature embryos are unable to retain a monosaccharide pool against a concentration gradient.

#### *The fate of assimilated monosaccharides*

The assimilation of monosaccharides, particularly during germination, must comprise an important part of embryo metabolism *in vivo*, hence pathways for the further metabolism and retention of accumulated glucose would be anticipated. In view of the instability of the 3-*O*-MG pool, it was of particular relevance to determine the fate of assimilated glucose.

Immature embryos were incubated with 100 mM [U- $^{14}$ C]glucose for 1 h and the molecular nature of the  $^{14}$ C-labelled pool investigated by paper chromatography and autoradiography. Although 450 nmol of glucose had been taken up per mg embryo d. wt, there was no detectable labelling of the glucose pool and the only  $^{14}$ C-labelled sugar was sucrose.

The rapid synthesis of sucrose from assimilated glucose in embryos is compatible with the importance of secondary metabolism in the energy-dependent uptake of hexoses.



*The specificity of monosaccharide uptake*

A characteristic of mediated transport is the specificity of the transmembrane carrier for a single defined group of substrates. If indeed a part of monosaccharide uptake in immature embryos is dependent on mediated transport, then some evidence of specificity could be expected.

The effect of 25 mM 2-deoxyglucose, a glucose analogue, on the initial rate of 3-O-M[U-<sup>14</sup>C]G uptake by embryos at substrate concentrations between 5 and 25 mM was determined (Fig. 5). At these low substrate concentrations 2-deoxyglucose appeared to act as a competitive inhibitor of 3-O-MG uptake.

Similar experiments designed to determine the effect of fructose on glucose uptake (results not shown) indicated that the uptake of aldo- and ketohexoses by embryos does not involve a common transport mechanism.

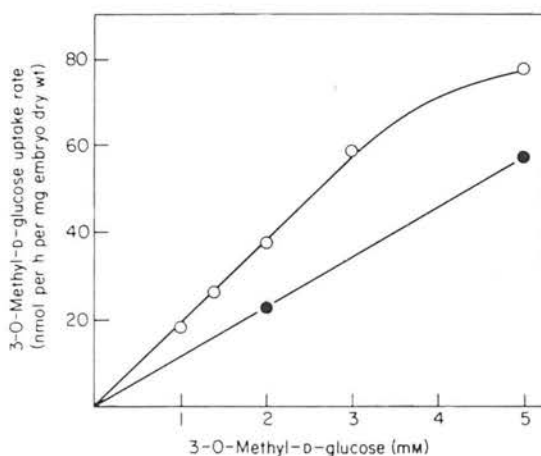


FIG. 5. The effect of 2-deoxyglucose on the kinetics of 3-O-methyl-D-glucose uptake by immature barley embryos. The initial rate of 3-O-methyl-D-(U-<sup>14</sup>C) glucose uptake by immature embryos (average length 1.73 mm) was determined at a range of substrate concentrations with (●) or without (○) added 25 mM 2-deoxyglucose in the standard assay medium as described in Fig. 1.

## DISCUSSION

The characteristics of monosaccharide accumulation in barley embryos have been investigated in isolated immature embryos *in vitro*. Both glucose and fructose are rapidly assimilated, and at substrate concentrations of 100 mM their rate of uptake exceeds that of sucrose by threefold. The relative rates of monosaccharide and disaccharide uptake are very similar to those determined in maize scutellum slices (Table 4).

The kinetics of glucose and fructose uptake with respect to increasing substrate concentration are not truly representative of Michaelis-Menten kinetics and it could be inferred that both passive diffusion and a saturable mediated process are involved in their uptake. In both maize (Whitesell and Humphreys, 1972) and barley embryos the  $K_m$  for monosaccharide uptake is high (> 100 mM) indicating the high permeability of the plasma membrane of scutellum cells to these sugars. From the inhibition of glucose uptake by uncouplers of oxidative phosphorylation, namely > 50 per cent by 0.1 mM 2,4-DNP and 1 mM sodium azide, it could be deduced that hexose uptake in barley embryos is either coupled directly to an active transport process or indirectly to a metabolic pathway whereby the depletion of the hexose pool would enhance glucose influx.

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TABLE 4. Comparative rates of sugar uptake by immature barley embryos and maize scutellum slices in vitro

Substrate (100 mM)	Initial rate of sugar uptake $\mu\text{mol per h}$ per g embryo f. wt	
	Barley embryos*	Maize scutellum slices†
Glucose	83	78
Fructose	89	78
Sucrose	36	41

\* Data from Fig. 1. † Data from Whitesell and Humphreys (1972).

In order to study the energetics of hexose uptake in isolation from its subsequent metabolism in barley embryos, the uptake of the glucose analogue 3-*O*-MG was investigated. 3-*O*-MG is accumulated by embryos and within the duration of most experiments could be regarded as a non-metabolite. The linear kinetics of 3-*O*-MG uptake, combined with the rapid efflux of 86 per cent of the 3-*O*-MG pool observed on dilution of the embryo bathing medium, indicated that simple diffusion must account for a large part of hexose uptake. Good evidence that secondary metabolism plays an important part in hexose assimilation in barley embryos was obtained from analysing the fate of accumulated glucose. After a period of rapid [ $^{14}\text{C}$ ]glucose uptake no detectable labelling of the glucose pool was observed and all the  $^{14}\text{C}$  label in the 80 per cent ethanol-soluble fraction was found in sucrose.

While the kinetics of 3-*O*-MG influx and efflux indicated that diffusion alone is responsible for hexose uptake in barley embryos, the uncoupling agents 2,4-DNP, FCCP (carbonyl cyanide *p*-trifluoromethoxyphenol hydrazone) and sodium azide were found to inhibit 3-*O*-MG uptake by 30–40 per cent. These results indicated that a low capacity active transport process may contribute to hexose uptake, whose energetics had been masked by the preponderance of hexose diffusion. This interpretation is further supported by the demonstration of competitive inhibition between the transport of the two glucose analogues 3-*O*-MG and 2-deoxyglucose. If a carrier protein is involved in glucose transport then it is specific for aldohexoses since there is no evidence of competition between glucose and fructose.

*In vitro* studies of the carbohydrate metabolism of wheat (Edelman, Shibko and Keys, 1959), maize (Whitesell and Humphreys, 1972; Humphreys, 1974), soya bean (Wahab and Burris, 1975*a, b*) and castor bean (Komor, 1977) seedlings have shown that glucose permeates the embryo by passive diffusion and is predominantly consumed in the synthesis of sucrose and phosphorylated derivatives. Imbibed wheat embryos incubated with [ $^{14}\text{C}$ ]glucose for 3 h were shown to incorporate 57 per cent of the label into sucrose, 29 per cent into sugar phosphates and 6 per cent into macro-molecular material (Edelman *et al.*, 1959). Whitesell and Humphreys (1972) have postulated that in maize scutellum hexose uptake is mediated by energy-dependent group translocation with the accumulation of hexose-6-phosphate in combination with passive diffusion. Sucrose synthesized from accumulated glucose forms a stable pool in the vacuole, while hexose phosphate pools in the cytoplasm are lost by efflux on exposure to osmotic shock. Phosphorylated derivatives were detected when barley embryos were fed with 3-*O*-MG, and the involvement of phosphorylation in 3-*O*-MG uptake might explain its sensitivity to uncouplers. While there is no evidence that active hexose uptake in barley embryos is coupled to a sugar-proton symporter, as envisaged for sucrose transport in maize (Humphreys, 1978) and castor bean (Hutchings, 1978; Komor, 1977) seedlings, a vectorial group transfer reaction similar to that proposed for sugar transport in bacteria (Lüttge and Schnepf, 1976) could be involved.

The dependence of barley embryos on passive diffusion for the assimilation of monosaccharides is compatible with the relatively minor role of these sugars in the nutrition of developing embryos, and the considerable concentration of free hexoses built up by starch hydrolysis during germination, sufficient to drive hexose influx throughout early seedling growth. The rapid metabolism of accumulated hexoses must contribute to the maintenance of this concentration gradient. A high affinity hexose transport mechanism could be important in the first hours of germination when free sugar concentrations in the endosperm are at a minimum.

## ACKNOWLEDGEMENTS

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# Variation in Starch Granule Size Distribution and Amylose Content During Wheat Endosperm Development

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## ABSTRACT

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This study confirmed that the amylose content of endosperm starch increases during grain development in wheat. Changes in the percent amylose content of endosperm starch granules separated into different size classes were then followed over the developmental period. At all stages of development, the amylose content of the smaller granules, expressed as a percentage of their total starch content, increased as starch granule size

increased. Amylose content of the other separated size classes also increased somewhat in the later stages of development. This was most marked with those less than 7  $\mu\text{m}$  in diameter. The largest granules (25–30  $\mu\text{m}$ ) accounted for only 0.4% of total granule numbers at 14 days after anthesis but accounted for about 3.7% by 56 days. At all stages of development, more than 78% of the granules were less than 10  $\mu\text{m}$ .

Grain development in barley (Banks et al 1973), wheat (Matheson 1971), and maize (Boyer et al 1976) appears to be characterized by a steady increase in the amount of amylose synthesized, relative to the total amount of starch. In developing sweet corn (Duffus and Jennings 1978), this is considered to be due both to changes in the relative numbers of the different sizes of starch granules and to changes in their amylose content. A similar situation may exist in developing barley endosperm (Williams and Duffus 1977), where the starch composition of small and large starch granules varies independently during development. In starch granules from mature maize (Boyer et al 1976) and barley (Goering and De Haas 1974) and from immature sweet corn (Duffus and Jennings 1978) and barley (Williams and Duffus 1977) endosperms, amylose content decreases as granule size decreases. However, in wheat at maturity (Bathgate and Palmer 1972) there was little difference in amylose content between large and small granules.

This article discusses the changes in starch granule composition and in relative numbers of the different size classes in relation to the reported (Matheson 1971) increase in amylose content during wheat endosperm maturation.

## MATERIALS AND METHODS

### Plant Material

Wheat (*Triticum aestivum* L.) var. Maris Huntsman was grown under commercial conditions on the University of Edinburgh farms. Ears were harvested at 14, 21, 25, 32, 40, 49, and 56 days after anthesis and stored at  $-18^{\circ}\text{C}$  until use.

### Fractionation of Starch Granules

The outer layers of pericarp and the embryo were removed from intact grains. The resulting endosperms were then suspended in ice-cold 20% (w/v) sucrose solution. The number of endosperms used varied from 20 in 4.0 ml of 20% sucrose at 14 days after anthesis to 5 in 40 ml at 56 days. Endosperms were homogenized manually in a Pottertype all-glass, ice-cooled homogenizer. After filtration through two layers of muslin, the filtrate was centrifuged at  $10,000 \times g$  for 10 min. The pellet, referred to as an unfractionated pellet, consisted of starch granules of all sizes. It was resuspended in 2.0 ml of 20% sucrose, and 1.0 ml of this sucrose was layered on top of two discontinuous sucrose gradients, each consisting of 2.0 ml each of 70, 65, and 50% sucrose in a 15-ml polypropylene centrifuge tube. This was centrifuged for 10 min at  $500 \times g$  in a swing-out head. After centrifugation, the starch granules could be seen as thin white layers concentrated at the interfaces and additionally as a pellet at the bottom. The layers were removed using a Pasteur pipette. The two pellets were resuspended in 1.0 ml each of 70% sucrose, layered on two further gradients of 2.0 ml each of 90, 85, 80, and 75% sucrose, and centrifuged as above.

The layers between 50 and 65% sucrose and between 65 and 70% sucrose were combined (size class 1). Similarly, the layer between 70 and 75% sucrose is size class 2; that between 75 and 80%, size class 3; those between 80 and 85% and between 85 and 90%, size class 4; and the combined pellets, size class 5.

After dilution with an equal volume of distilled water and sedimentation of the starch granules by centrifugation, the pellets were washed four times in ice-cold distilled water followed by centrifugation. This removed all soluble carbohydrates. Because the time between sampling and obtaining the initial washed pellet generally was not more than 20 min and because all operations were performed at about  $2-4^{\circ}\text{C}$ , any starch degradation by amylase contamination was assumed to be minimal. No increase in reducing sugar content of washed fractions was observed with time.

Fractions were combined in order to reduce the numbers for subsequent analysis. Final fractions were not a quantitative recovery of all starch granules of a single size but were representative of all starch granules in that size range.

### Starch Granule Number

The method was a modification (Duffus and Jennings 1978) of that described by May and Buttrose (1959). Only granules stained with iodine were counted.

### Starch Granule Size

Starch granules were stained with iodine and examined under a magnification of 1,000 diameters. Measurements of size were made with an ocular micrometer. Samples contained between  $1.7 \times 10^3$  starch granules (14 days) and  $3.5 \times 10^3$  starch granules (49 days).

### $\alpha$ -Glucan and Amylose Determinations

Total  $\alpha$ -glucan was determined in starch granule fractions using the anthrone-sulfuric acid reagent (McCready et al 1950) and amylose by the method of Gilbert and Spragg (1964). Amylose content was determined by reference to a standard curve using pure amylose. All results were averages of three separate experiments, that included duplicate amylose and total  $\alpha$ -glucan determinations.

### Scanning Electron Microscopy

Starch granule fractions and an unfractionated pellet from wheat endosperm 27 days after anthesis were prepared for scanning electron microscopy. After dehydration in an acetone series, the granules were mounted on stubs by means of double-sided sticky tape, then coated with gold, and examined in a Cambridge stereoscan electron microscope.

## RESULTS AND DISCUSSION

The amylose content of the unfractionated starch granules increased steadily over the developmental period (Table 1), thus confirming previous results (Banks et al 1973, Boyer 1976, Matheson 1971).

Starch granules from the sucrose gradients increased in size as



the sucrose density increased. Dimensions of all size classes are shown in Table II. A range of sizes rather than a mean diameter is given in order to emphasize that the fractions are not uniform. Some overlap was observed between size classes 1 and 2. Significant numbers of granules were obtained in each size class throughout the development period (Table III), indicating that the two types of granules exist in a continuous range of sizes in wheat.

Scanning electron micrographs (Fig. 1) showed that the starch granules were undamaged in the isolation procedure and that each size fraction fell broadly within the size ranges as estimated by light microscopy. The smallest size fraction (Fig. 1B) contained a mixture of spherical ( $\alpha$ -type) and small lenticular ( $\beta$ -type) granules. These  $\beta$ -type granules may be precursors of the large granules more clearly seen in size class 5 (Fig. 1F). This suggests that pure fractions of the  $\alpha$ -type granules may be impossible to obtain.

The amylose content of the smaller starch granules was less than that of the larger granules at all stages of development (Table II), confirming previous results (Boyer et al 1976, Duffus and Jennings 1978, Goering and De Haas 1974, Williams and Duffus 1977). This finding is not surprising, at least at early stages, because the small starch granules are presumably synthesized first and therefore account for the major amount of polysaccharide present at that time. In wheat, barley, and maize, the polysaccharide is presumably amylopectin.

The increase in amylose content of whole grain starch is clearly not caused by continuous change in the amylose content of

granules with identical starch compositions but is caused by variation in the rates of appearance of granules with different starch compositions. As can be seen in Tables I and II, relatively more of the granules in size class 1 must account for an overall amylose content of 6% at 14 days, and relatively more of the granules in size class 5 must account for an overall amylose content

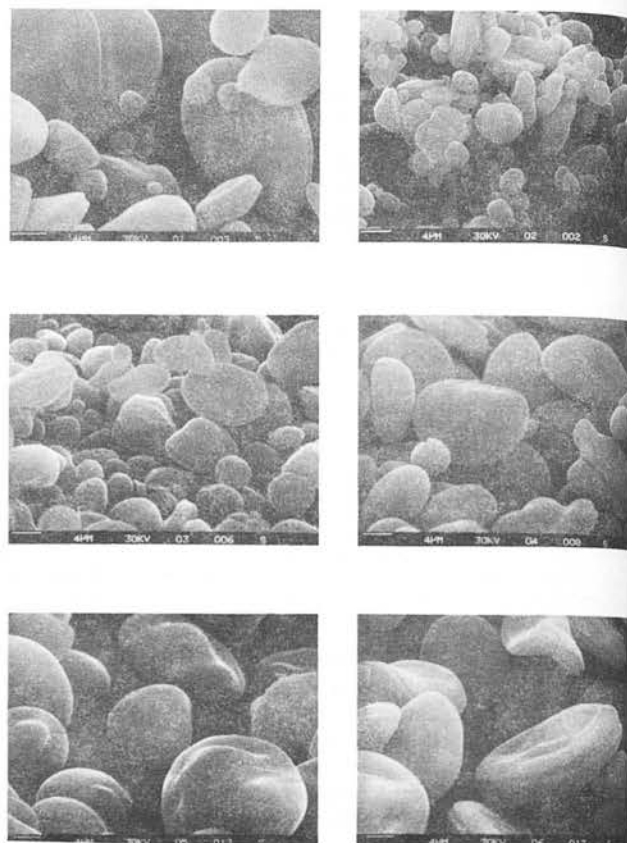


Fig. 1. Scanning electron micrographs of wheat endosperm starch granules at 27 days after anthesis, before and after separation on sucrose density gradients. Bar markers represent 4  $\mu$ m. A, unfractionated pellet; B, size class 1; C, size class 2; D, size class 3; E, size class 4; F, size class 5.

TABLE I  
Changes in Amylose Content of Unfractionated Starch Granules during Endosperm Development in Wheat<sup>a</sup>

Days After Anthesis	Amylose Content of Starch Granules (% w/w)	Total $\alpha$ -Glucan in Starch Granules (mg/endosperm)
14	6.0 $\pm$ 1.1	2.5 $\pm$ 0.5
21	8.5 $\pm$ 1.9	3.1 $\pm$ 0.9
25	10.2 $\pm$ 4.1	13.1 $\pm$ 3.5
32	13.7 $\pm$ 2.1	22.2 $\pm$ 1.6
40	18.3 $\pm$ 1.4	19.2 $\pm$ 1.4*
49	22.8 $\pm$ 2.3	29.6 $\pm$ 2.0
56	28.3 $\pm$ 5.4	27.7 $\pm$ 2.5

<sup>a</sup> Results are the mean  $\pm$  standard deviation.

TABLE II  
Amylose Content as % (w/w) of total  $\alpha$ -Glucan in Starch Granules of Different Size Classes during Endosperm Development in Wheat<sup>a</sup>

Size Class	Size Range ( $\mu$ m)	Age in Days After Anthesis						
		14	21	25	32	40	49	56
1	2-7	11.5 $\pm$ 4.5	11.2 $\pm$ 1.6	11.5 $\pm$ 4.6	10.0 $\pm$ 3.8	11.2 $\pm$ 2.5	13.4 $\pm$ 4.1	17.8 $\pm$ 3.1
2	5-10	13.4 $\pm$ 7.1	14.5 $\pm$ 6.0	13.6 $\pm$ 6.7	14.9 $\pm$ 5.7	16.5 $\pm$ 2.9	14.5 $\pm$ 7.7	17.7 $\pm$ 1.9
3	10-15	21.1 $\pm$ 5.8	12.0 $\pm$ 3.1	12.3 $\pm$ 1.2	16.8 $\pm$ 2.1	19.3 $\pm$ 1.7	17.1 $\pm$ 1.9	23.8 $\pm$ 1.4
4	15-25	20.4 $\pm$ 6.8	21.6 $\pm$ 4.8	17.5 $\pm$ 0.2	17.5 $\pm$ 4.3	15.5 $\pm$ 3.3	22.0 $\pm$ 8.5	28.2 $\pm$ 6.1
5	25-30	21.8 $\pm$ 7.5	25.4 $\pm$ 5.7	19.7 $\pm$ 1.3	22.6 $\pm$ 3.1	20.0 $\pm$ 0.2	22.7 $\pm$ 3.1	30.8 $\pm$ 7.3

<sup>a</sup> Results are the mean  $\pm$  standard deviation.

TABLE III  
Percentage of Total Numbers of Starch Granules in Each Size Class During Endosperm Development in Wheat<sup>a</sup>

Size Class	Size Range ( $\mu$ m)	Age in Days After Anthesis						
		14	21	25	32	40	49	56
1	2-7	64.1 $\pm$ 3.9	76.0 $\pm$ 2.7	75.9 $\pm$ 5.2	68.0 $\pm$ 5.2	78.2 $\pm$ 2.2	77.7 $\pm$ 1.7	76.2 $\pm$ 2.6
2	5-10	14.1 $\pm$ 1.7	8.25 $\pm$ 1.8	8.6 $\pm$ 1.8	12.7 $\pm$ 2.3	13.6 $\pm$ 1.7	13.1 $\pm$ 1.5	11.7 $\pm$ 1.5
3	10-15	10.7 $\pm$ 1.8	6.0 $\pm$ 1.3	6.0 $\pm$ 0.9	7.4 $\pm$ 2.3	3.0 $\pm$ 1.0	3.3 $\pm$ 0.6	4.7 $\pm$ 0.9
4	15-25	10.6 $\pm$ 1.9	8.1 $\pm$ 1.1	7.4 $\pm$ 2.0	7.7 $\pm$ 1.6	3.5 $\pm$ 0.7	3.6 $\pm$ 0.7	3.5 $\pm$ 0.5
5	25-30	0.4 $\pm$ 0.2	1.6 $\pm$ 0.4	2.1 $\pm$ 0.8	4.1 $\pm$ 0.9	1.6 $\pm$ 0.5	2.4 $\pm$ 0.5	3.7 $\pm$ 1.1

<sup>a</sup> Results are the mean  $\pm$  standard deviation.

of 28.3% at 56 days. Although the numbers in size class 5 do increase from 0.4 to 3.7% during the developmental period (Table III), the relative numbers in size class 1 also increase. The difference is made up by decreases in the relative numbers of granules in size classes 3 and 4.

At 14 days a major contribution to grain starch is made by the smallest granules, which have the lowest amylose content but account for over 64% of total numbers. In sweet corn, granules less than 1.4  $\mu\text{m}$  account for about 70% of total numbers at this age (Duffus and Jennings 1978). The relative contributions of A-type (0-8  $\mu\text{m}$ ) and B-type granules (8-26  $\mu\text{m}$ ) to total starch weight in wheat var. Maris Huntsman at maturity vary according to grain size and degree of grain filling (Brocklehurst and Evers 1977), which are influenced by differences in rates and conditions of grain growth. In all types of grain, however, the percentage by weight of granules under 10  $\mu\text{m}$  was about 40%. Certainly our results show clearly that the small granules make some contribution to total wheat starch because, at maturity, about 88% of the total number of granules is made up of those less than 10  $\mu\text{m}$  in diameter. In sweet corn, however, the smallest granules contribute less than 4% of total starch but account for as much as 50% of total numbers (Duffus and Jennings 1978), and in barley (Bathgate and Palmer 1972) at maturity the small granules are 88% of total numbers but contribute only 11% of the starch. Although in maize, the small granules are thought to derive from younger cells (Shannon 1974), the results reported here are difficult to relate to their likely physiological origins because the smaller granules also may occur in large numbers in the interior and hence older cells of the endosperm (Sandstedt et al 1968).

In addition to progressive increase in amylose content as granule size increases, amylose content also may increase in the separated size classes as they mature. This is clearest in size classes 1 and 5 between 40 and 56 days after anthesis. The increasing amylose content of size class 1 as maturity approaches may account for Bathgate's observation (1972) that, composition differs little between granules divided into small and large fractions.

We conclude that the increase in percent amylose of endosperm starch relates mainly to changes in relative numbers of the different size classes present, which show increasing amylose content with

increasing size. Changes in the amylose content within a size range, particularly size class 1 (A-type granules), also contribute to the overall increase in percent amylose content of total starch.

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## CHAPTER 9

**Carbohydrate Metabolism and Cereal Grain Development**

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## I. INTRODUCTION

The changes which accompany cereal grain development have been subject to increasing attention from plant physiologists and biochemists in the past ten years. This is partly in response to a requirement for identifying those factors concerned in the regulation of grain yield and composition, and partly due to interest in a system which is now known to be of extreme complexity. This chapter will review the present information about carbohydrate metabolism during grain development and maturation in a range of cereals, including barley, wheat, rice and maize.

## II. MORPHOLOGICAL CHANGES DURING GRAIN DEVELOPMENT

### A. OVERALL CHANGES

Since the pioneering work of Buttrose (1963) few attempts have been made to describe the ultrastructural changes accompanying cereal grain development. This may be due in part to the technical difficulties involved in obtaining good sections of a tissue, which varies from a semi-liquid at early stages to a hard, sometimes flinty tissue of low moisture content at maturity; and in part to problems in obtaining fresh tissue at all stages of development. On the other hand the initial events following fertilization have been well described using light microscopy (Brink and Cooper, 1947; Luxová, 1967). The pollen germinates on the stigma and the two male gametes carrying sister nuclei are carried to the ovary in the tip of the pollen tube. One nucleus enters the egg cell and the two nuclei unite to form the zygote. The second male gamete nucleus enters the central cell and unites with the two polar nuclei to form the primary endosperm nucleus. Rapid division of the triploid endosperm nucleus follows anthesis and proliferation of nuclei takes place, initially without accompanying cell wall formation. By about two to three days after anthesis in barley, when cell wall formation commences, there might be from 200–500 endosperm nuclei within the embryo sac (Thompson and Johnston, 1945). The rate of cell division in the embryo is rather less than in the endosperm and by ten days after anthesis in barley it is about 0.6 mm long and accounts for around 3% of the total grain fresh weight. Ultrastructural changes during endosperm development in barley (Buttrose, 1960) and wheat (Buttrose, 1963) have been described in some detail. Mitochondria, proplastids, endoplasmic reticulum and Golgi bodies were distinguishable from five days after anthesis. Starch deposition within amyloplasts commences early in development as does the formation of small spherical and oval membrane-bound granules which may be protein bodies (Fig. 1). Many of the mitochondria are grouped around the immature amyloplasts (Fig. 2) (Williams and Duffus, 1978). In maize however, amyloplasts appear rather later in development (Boyer, 1977). Surrounding the endosperm, with the exception of the area adjacent to the embryo, is a specialized layer of cells called the aleurone. These can first be distinguished in wheat about ten days after anthesis and are then cuboidal in shape with thin walls and large nuclei (Morrison *et al.*, 1975). Starch granules are present early on but disappear by 21 days after anthesis. At maturity the cytoplasm is closely packed with aleurone grains (protein granules) completely surrounded by lipid droplets. There are numerous mitochondria even at this stage. A developmental study with maize aleurone showed some similarity to wheat but there was little evidence of the presence of significant numbers of starch granules (Kyle and Styles, 1977). Embryo development, recently reviewed by Raghavan (1976), is

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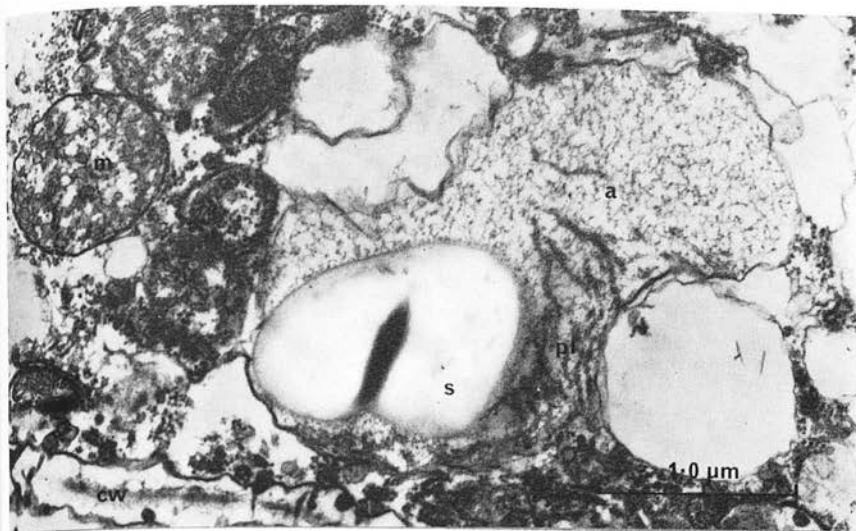


FIG. 1. A section through an endosperm of *H. distichon* L. var. Julia half-way down the grain 18 days after anthesis. a, amyloplast; s, starch; cw, cell wall; pl, plastid lamellae; m, mitochondrion. The tissue was fixed in glutaraldehyde, post-fixed in osmium tetroxide and sections were stained in uranyl acetate followed by lead citrate. (Micrograph supplied by M. P. Cochrane.)

characterized by the appearance of a variety of storage proteins, amyloplasts, extensive endoplasmic reticulum, mitochondria and large numbers of ribosomes.

Complex structural changes also occur in the maternal tissues surrounding the endosperm and embryo during the developmental period. In barley, wheat, oats and rye these include the testa, the pericarp and various tissues such as the glumes and paleae which later become the husk. Initially these tissues, with the exception of the testa, contain chloroplasts and make up about 70–80% of the grain fresh weight. Their relative contribution to grain weight decreases throughout development until at maturity they form a tough highly compressed layer surrounding the grain. The maize ear, on the other hand, is a spike with a thickened axis containing many hundreds of grains. The ear develops within a multilayered series of short pale green leaflike structures. Unlike the other cereals, the pericarp, which in maize forms a tough protective cover even at early stages of development, does not have a chlorophyll-containing layer.

Since it appears that developing wheat grains (Evans and Rawson, 1970) may themselves account for a substantial amount of gross ear photosynthesis, interest has been focused recently on the structural (Morrison, 1976) and functional (Nutbeam and Duffus, 1976) properties of the pericarp. Morrison (1976) has described the ultrastructural changes taking place during wheat grain development in the chlorophyll-containing cross cells and tube cells of the inner pericarp. In the developing tube cells amyloplasts containing starch

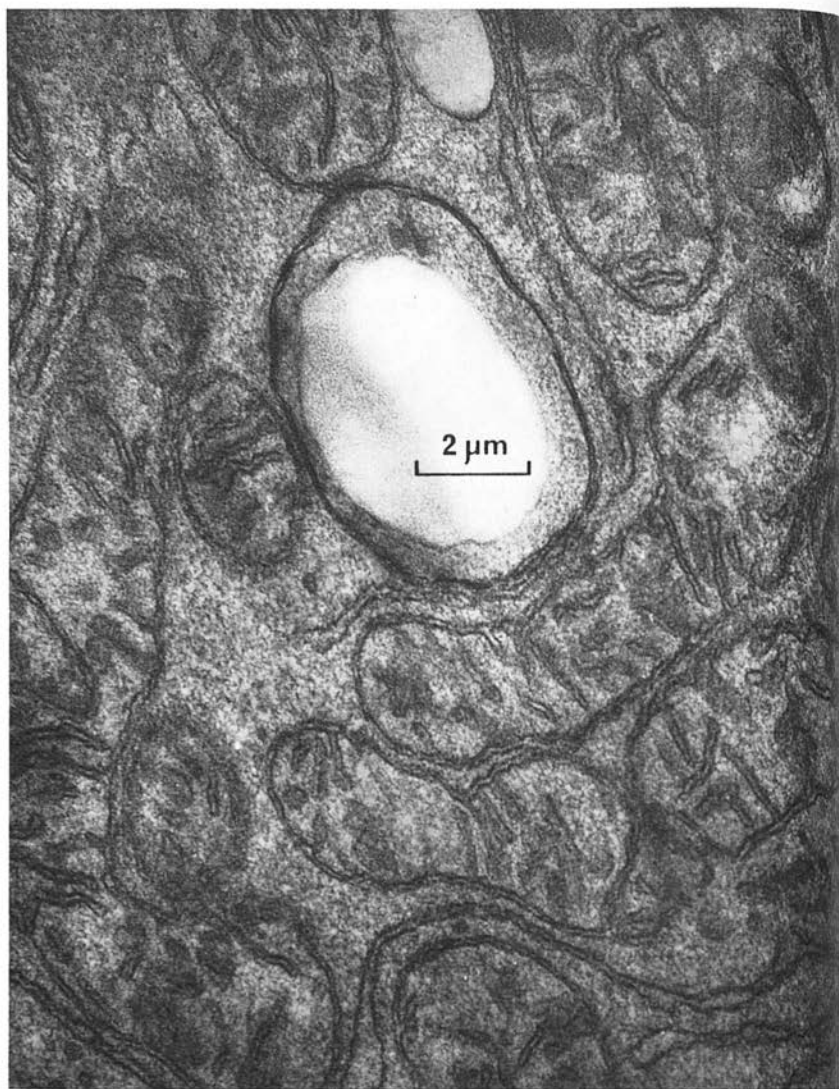


FIG. 2. Mitochondria surrounding amyloplast in immature barley endosperm. The tissue was fixed in potassium permanganate. (Williams and Duffus, 1978.)

granules are seen as early as three days after anthesis. By ten days after anthesis starch is present in the chloroplasts of both types of cell and persists in the cross cells until degeneration sets in and the grain turns from bright green to yellow. Similar observations (Fig. 3) have been made for barley (M. P. Cochrane, in preparation).

It is interesting to note the observation of MacGregor *et al.*, (1972) that at anthesis the outer non-chlorenchymatous layer of the pericarp contains large

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FIG. 3.  
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numbers of small starch granules which disappear about ten days after anthesis. A diagrammatic representation of the outer layers of a mature wheat grain is shown in Fig. 4.

It is worth noting in this context that since maturation periods vary according to climate and species, age in days after anthesis is rarely a true measure of development. For example, in Britain the period between anthesis and maturity for barley is around 60 days whereas in the Canadian prairies it is around 40 days. Thus a system which relates morphological stage, as measured by such parameters as cell number, amyloplast size or simply dry weight, to an arbitrary scale of age in days after anthesis is the optimal method and one which is in fact probably used in many laboratories. Cerning and Guilbot (1973) have expressed a number of their results as a function of a climatic factor. The sole climatic variable used was temperature. Until it is shown how this and other variables are related to the rate of growth and differentiation, such potentially useful techniques will not be universally adopted.

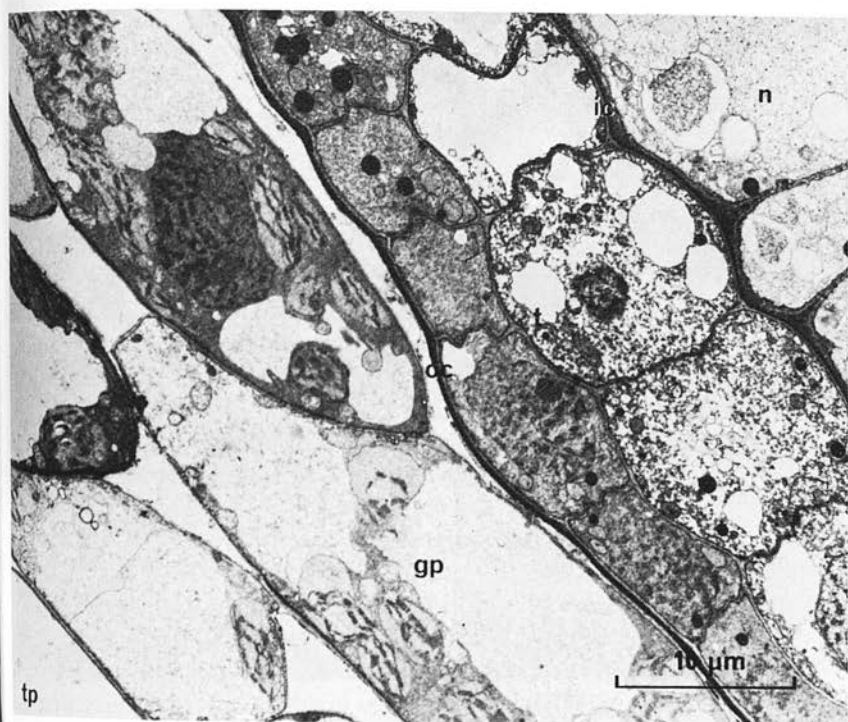


Fig. 3. Transverse section through the upper part of the dorsal side of a grain of *H. distichon* L. var. Julia fixed 20 days after anthesis. n, nucellar epidermis; t, testa; gp, green layer of pericarp; tp, transparent layer of pericarp; oc, outer cuticle; ic, inner cuticle. The tissue was fixed as described in Fig. 1. (M. P. Cochrane, 1979.)

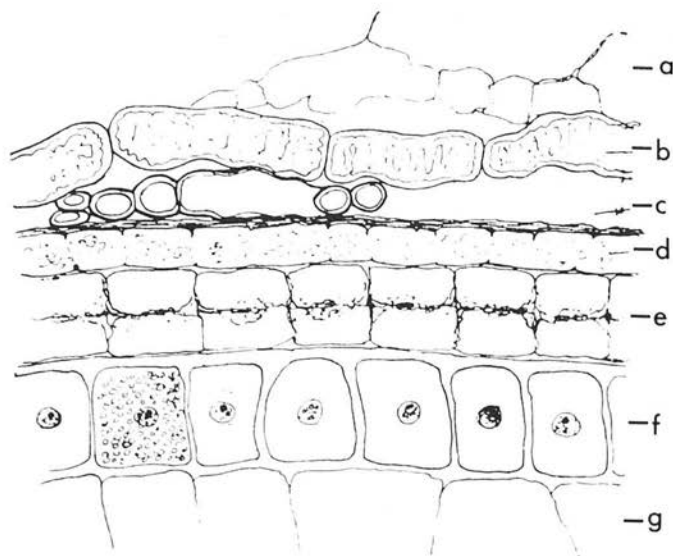


FIG. 4. Transverse section through a mature wheat grain illustrating the arrangement of the outer layers. a, transparent layer of pericarp; b, green layer of pericarp; c, tube cells; d, testa; e, nucellar epidermis; f, aleurone; g, endosperm (modified from Percival, 1921).

#### B. AMYLOPLAST DEVELOPMENT

The mature amyloplast is an organelle surrounded by a double membrane and containing one or more starch granules. Frequently there is confusion, in particular with isolated fractions, as to whether one is dealing with amyloplasts or starch granules, since it is not always easy to verify the presence of a surrounding double membrane. Additionally, it is not always clear that the starch granules being considered have not been degraded during purification and are in fact small fragments of the original. Such points must be kept in mind when considering the following results.

Starch granules from mature wheat and barley endosperms can be divided into two populations, large and small. Evers (1971), using the scanning electron microscope, has described the development of the large or A-type starch granules in wheat. These are lenticular in shape and have a peripheral groove (Fig. 5). The smaller—Type B—are more numerous and differ in starch composition from the large (see Section III D): they may well, therefore, have different biological origins.

There is some controversy surrounding the initial stages of amyloplast development. Buttrose (1963) has stated that in wheat only one starch granule is initiated per amyloplast whereas Williams and Duffus (1978) have presented evidence for multiple initiation sites. May and Buttrose (1959) have suggested that the large starch granules are initiated up to two weeks after anthesis and the small only after 14 days. These proposals have been

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confirmed (Williams and Duffus, 1978) by fractionating amyloplasts at different stages of development which are labelled with  $^{14}\text{C}$  derived from flag leaf photosynthesis. Since the small amyloplasts were labelled only at 14 and 24 days after anthesis, whereas the large were labelled throughout development, it was concluded that the small amyloplasts were initiated only during this period of development. It seems likely that starch granules are initiated within a group of the many endosperm proplastids seen soon after anthesis. The suggestions of Badenhuizen (1958) that amyloplasts are derived from mitochondria, and Buttrose (1960), that small amyloplasts are budded off from the large, have not so far been confirmed by other workers.

Studies of the developing maize endosperm indicate that it is composed of a population of cells of varying physiological ages (Lampe, 1931). While there is no doubt that some of the small starch granules are derived from young cells (Boyer *et al.*, 1976a), small granules are also found mixed in with the large granules in the interior of the endosperm (Sandstedt *et al.*, 1968). Maize has in

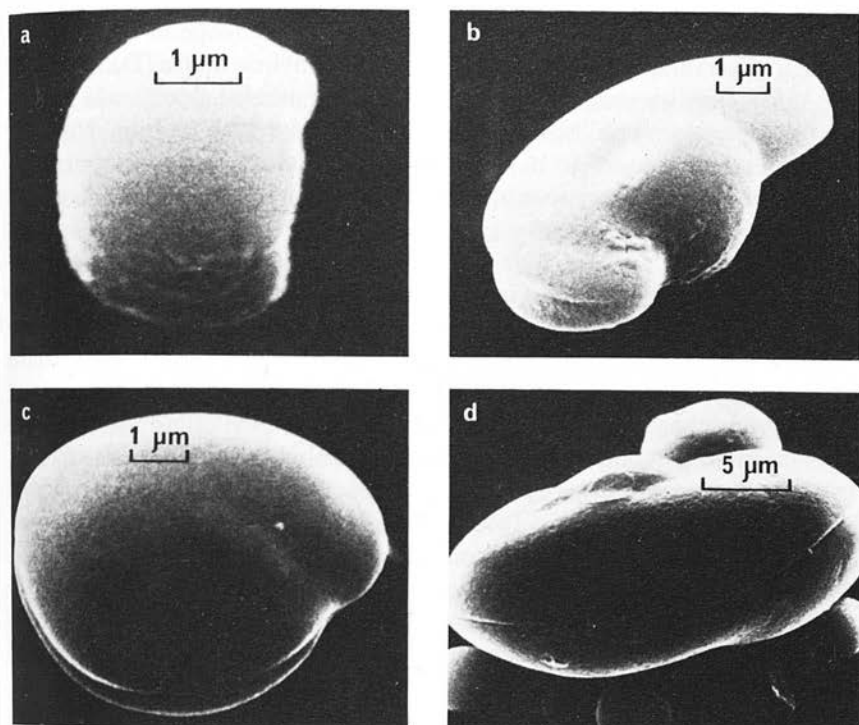


FIG. 5. Scanning electron micrographs of large starch granules isolated from wheat endosperm at different stages of development. a. 4 days after anthesis; b. 10 days; c. 12 days; d. 52 days. Initially the shape is spherical (a) and becomes progressively surrounded by a lip-like structure with a groove around the equatorial circumference (b). More mature granules have a pronounced bulge on the upper surface (c) and finally assume the lenticular shape of the mature granule (d). (Evers, 1971). Reproduced by permission of Cambridge Scientific Instruments.

addition to the normal genotype, a wide range of mutant genotypes which affect kernel polysaccharides. These include *brittle-1*, *brittle-2*, *dull*, *shrunk-1*, *sugary-1*, *waxy* and *amylose-extender*. Many or most of these genotypes have an extraordinary range of odd-shaped starch granules (Sandstedt, 1965). The development of some of these has been described by Boyer *et al.*, (1976b) using light microscopy. Normal endosperm cells undergo a developmental sequence of nuclear enlargement, initiation of small spherical starch granules closely associated with the nucleus, granule movement away from the nucleus and continued granule enlargement to maturity. On the other hand, in *amylose-extender* granule movement away from the nucleus is followed by a secondary initiation of abnormal granules in close association with the nucleus. Endosperm development in *sugary* is characterized by the appearance of large rounded phytyglycogen plastids together with other large amyloplasts which themselves contain smaller starch granules.

An abnormal pattern of starch granule development has been observed (Merritt and Walker, 1969) in the high amylose barley designated Glacier (Pentlandfield) where the average diameter of the starch granules was smaller than those of the normal varieties at all stages of endosperm development. Similar observations have been made for high amylose maize (Deatherage *et al.*, 1954). High amylose barley was originally of interest since it was thought that the granules would be more readily degraded during malting. However, this mutant has proved to be more resistant to its own enzyme system and gives lower extracts than normal varieties in micro-malting tests (Ellis, 1976).

### III. VARIATION IN GRAIN CARBOHYDRATE COMPOSITION

#### A. SUPPLY OF CARBOHYDRATES TO THE DEVELOPING GRAIN

The dry matter entering the barley grain is derived from photosynthesis after ear emergence (Archbold, 1942) and it seems probable that this is also the case in wheat. An appreciable proportion (76%) of grain dry weight is derived from the ear itself (Frey-Wyssling and Buttrose, 1959), and in wheat (Evans and Rawson, 1970) the grain can account for 40% of gross ear photosynthesis which is presumably derived from carbon dioxide fixation in the chlorophyll-containing layers of the pericarp. Analysis of the exact composition of the soluble carbohydrate fraction entering the grain has not yet proved possible since it is likely that this travels in the phloem, a tissue notoriously difficult to sample. Sucrose is generally considered to be the main transport material in higher plants and, while glucose, fructose, fructans and other soluble carbohydrates are found in leaves and stems of cereals and grasses, they were thought unlikely to be involved in transport or even as direct sources of carbon for seed production (Porter, 1962). For example Mayer and Porter (1960) have examined movement of carbohydrate at anthesis in rye by

supplying single leaves with  $^{14}\text{CO}_2$  and determining the distribution of radioactivity in sucrose and fructose polymers throughout a tiller. Although most of the radioactivity in leaf extracts was in sucrose all the fructose polymers were radioactive. In sheaths and internodes, however, activity was confined to sucrose and no trace of activity was found in the large array of water-soluble fructans found in these tissues. Thus it was concluded that sucrose was the only labelled product moving from the site of assimilation at the grain-filling stage. The end product of pericarp photosynthesis is sucrose (Nutbeam and Duffus, 1976) and it is assumed in the discussion which follows, that carbohydrate arrives at the grain mainly as sucrose.

The levels of carbohydrate entering the grain are not necessarily the same as those in the phloem, since Jenner and Rathjen (1975) have reported that in wheat a mechanism may operate which limits the supply of sucrose to the developing grain. Thus, while supplies of carbohydrate may be adequate, factors such as a fall in the capacity of the grain to utilize sucrose for starch synthesis may limit its accumulation.

#### B. SUGARS

In order to understand the control mechanisms associated with any metabolic system it is essential to determine *in vivo* concentration levels, and intracellular location of both substrates and products simultaneously with any measurements of relevant enzyme activity. However, in much of the work concerned with the biochemistry of development in cereal grains such measurements have not been made. Additionally, the types of sugars present, together with their relative concentrations will influence the chemical composition and hence the market value of the final product. Early studies (Harris and MacWilliam, 1957) indicated that the principal sugars in the leaves and stems of barley are found in whole grain from the earliest stage of development. Initially fructan was found to predominate, followed by sucrose, glucan, "glucodiffructose", fructose, glucose and maltose. Levels fell after the maximum at 28 days after anthesis. Raffinose, which is apparently located uniquely in the embryo, did not appear until some six or seven weeks after anthesis (Fig. 6).

More recent work with developing wheat and barley grains confirms these observations. For example it has been shown that barley endosperm reducing sugars (Jennings and Morton, 1963; Baxter and Duffus, 1973a) and, indeed, total sugars (MacGregor, *et al.*, 1971) of intact kernels fall from early maximum levels as starch accumulates. In wheat kernels similar results were obtained (Abou-Guendia and D'Appolonia, 1973a; Cerning and Guilbot, 1973; Kapoor and Heiner, 1976). In both wheat and barley sucrose levels were generally about 33% of total alcohol-soluble sugar levels. Fructose and fructans were also present in appreciable amounts early in development, and in wheat levels initially were greater than those of sucrose (Cerning and

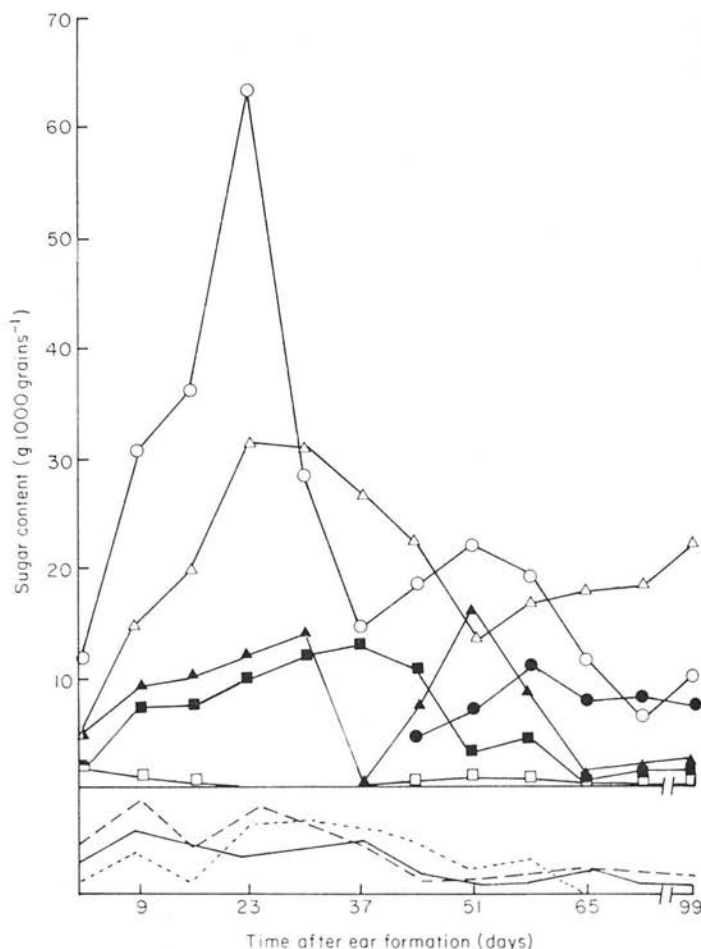


FIG. 6. Variation in amount of sugars during grain development in barley.  $\circ$ , fructan;  $\Delta$ , sucrose;  $\blacktriangle$ , glucan;  $\blacksquare$ , glucodifructose;  $\bullet$ , raffinose;  $\square$ , galactan. Lower graph: ----, fructose; —, glucose; ·····, maltose (Harris and MacWilliam, 1957).

Guilbot, 1973). In rice (Akazawa *et al.*, 1964) glucose and fructose levels were one third of sucrose levels and a similar decrease for these was observed from an early maximum. Since the amounts of glucose and fructose agreed closely with total amounts of non-reducing and reducing sugars it was concluded that no significant amounts of other sugars were present.

In general, therefore, mono-, di- and oligo-saccharide levels decrease from early maximum levels during grain maturation. Keeping in mind that most of the above results refer to intact grain, the additional conclusion might be that starch is then synthesized at the expense of these sugars. However, the explanation can hardly be this simple since the accumulation of starch is so much greater than the decrease in levels of reducing sugars. No doubt a

mechanism exists whereby the concentration of precursors per cell is maintained at the levels required for starch synthesis. Thus, in this connection measurements of the rates of reducing sugar and sucrose turnover would be of more value than measurement of total sugars.

### C. NON-STARCH POLYSACCHARIDES

The principal polysaccharides of the barley grain, other than starch and fructan, are cellulose and hemicelluloses. Cellulose is confined mainly to the outer layers of the grain whereas hemicelluloses can be found throughout (Harris, 1962).

While the composition of mature barley endosperm cell wall has been well described (Fincher, 1975), little is known of the biosynthetic processes involved. These cell walls have long been known to be exceptional among those of higher plants since they contain no pectin and very little cellulose (MacLeod and McCorquodale, 1958; MacLeod and Napier, 1959). They contain a microfibrillar phase embedded in an amorphous phase (Fincher, 1975). The microfibrillar phase is thought to consist of cellulose together with tightly bound arabinoxylan and polysaccharides rich in mannose. The matrix material is arabinoxylan (approximately 25%) and  $\beta$ -glucan (approximately 75%).  $\beta$ -Glucans are linear molecules with around 30%  $\beta$ - (1,3) and 70%  $\beta$ - (1,4) linkages generally randomly dispersed, and in the cell wall they are associated with firmly linked peptide sequences (Fleming and Kawakami, 1977; Forrest and Wainwright, 1977). Interest in endosperm cell wall synthesis stems from the fact that incomplete hydrolysis of  $\beta$ -glucan during malting can result in slow filtration and lead to haze formation in high gravity beers. MacLeod (1976) has suggested that these effects could be minimized by accelerating and enhancing the action of endo- $\beta$ -glucanases during malting or by selecting barleys with a minimum content of cell wall  $\beta$ -glucan. Since Palmer (1975) has shown that development of cell wall degrading enzymes during malting was equally rapid in a cultivar which modifies with difficulty, as in good malting cultivars, the second approach was considered (MacLeod, 1976) to be the more promising. However, little is known of the mechanism of cell wall synthesis during grain development and so far a successful screening procedure for selecting low  $\beta$ -glucan cultivars has not been developed (MacLeod, 1976).

While  $\beta$ -glucan deposition has been little investigated, some attention has been paid to the pattern of pentosan accumulation during grain development. In barley (Cerning and Guilbot, 1973) pentosans accumulate rapidly during the first 18 days of kernel development, while in wheat (Cerning and Guilbot, 1973; Abou-Guendia and D'Appolonia, 1973b) the rate of accumulation is rather less. Whole kernels were used and the proportion present in endosperm was not determined. Similar results were obtained by Jennings and Morton (1963) for isolated developing endosperms, and pentosan accumulation was

attributed to the synthesis of new cell walls. Certainly the results correlate quite well with the time of greatest cell wall proliferation in developing endosperm. In a comparison of bran and endosperm pentosans in mature and immature wheat, D'Appolonia and MacArthur (1976) found that while the pentosan content of endosperm decreased slightly in the 20 day period taken to reach full maturity from the preripe stage, the converse was observed for the outer layers. It may be possible that the precursors of bran pentosans originate in the endosperm.

#### D. CHANGES IN COMPOSITION OF STARCH GRANULES

The starch content of cereal grains increases steadily throughout development and generally accounts for around 65–75% of grain dry weight at maturity. It also seems to be generally true that the amount of amylose relative to the amount of starch increases throughout grain development. In barley, for example, the amylose content of total starch doubles during grain development (Banks *et al.*, 1973; Table I) and in wheat it increases from 14–20% between two and six weeks after anthesis (Matheson, 1971). The same is true of a number of maize genotypes which affect polysaccharide accumulation. Exceptions are the mutant *waxy* where the apparent amylose percentage remained near zero and the double mutant *amylose-extender waxy* where the percentage decreased during development (Boyer *et al.*, 1976a).

The increase in percentage amylose content may be caused by a steady change in the starch composition of all granules. On the other hand, it might be due to changes in relative numbers and/or changes in individual amylose

TABLE I  
The apparent amylose content of the starch from normal barley  
(Glacier CI 9676)

Maturity <sup>a</sup>	Amylose content (%) <sup>b</sup>
9	15.8
14	19.1
20	23.2
27	26.2
32	29.2
46	28.3

<sup>a</sup> Number of days from the average date of anthesis.

<sup>b</sup> Measured at 20.4 C by:

Amylose content (%) =

$\frac{\text{Iodine-binding capacity of starch} \times 100}{\text{Iodine-binding capacity of corresponding amylose}}$

(Data from Banks *et al.*, 1973).



contents of the amyloplasts of different types. The former may be the case in wheat (Bathgate and Palmer, 1972) since at maturity there is no significant difference in amylose percentage of the starch of the large and small granules. Both Matheson (1975) with *sugary*, and Boyer *et al.* (1976a) with *normal*, *amylose-extender* and the double mutant *amylose-extender sugary*, found that the largest granules had a significantly higher amylose content than the smallest. These differences are maintained during development in *sugary* (Duffus and Jennings, 1978) and in *normal* barley (Williams and Duffus, 1977). The low amylose content of immature *sugary* endosperm is reflected in the relatively greater proportion of small granules at that time, and the high amylose (23%) at maturity reflects the fact that nearly 60% of total  $\alpha$ -(1,4) (1,6) glucan is then found in the largest granules. Matheson (1975) has shown that the very small starch granules typical of *sugary* endosperms are made up of phytyglycogen and amylose. As granule size increases so does the molecular weight of the phytyglycogen. Thus it can be suggested that increasing molecular weight of synthesized  $\alpha$ -(1,4) (1,6) glucans may be a factor in the initiation of starch granule synthesis.

#### IV. DEVELOPMENTAL ENZYMOLOGY OF STARCH SYNTHESIS

##### A. SUCROSE METABOLISM

Sucrose can be metabolized in living tissues by a number of enzymes (Table II). Sucrose phosphate synthase (E.C.2.4.1.14) was considered by Akazawa (1972) not to be involved in sucrose metabolism during grain development, since the equilibrium lies in favour of sucrose phosphate synthesis and indeed, the reaction is essentially irreversible. Furthermore, its activity in developing

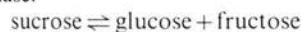
TABLE II  
Sucrose metabolizing enzymes

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Sucrose synthase:



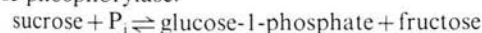
Invertase:



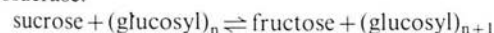
Sucrose phosphate synthase:



Sucrose phosphorylase:



Amylosucrase:



rice endosperm was found to be much less than sucrose synthase (E.C.2.4.1.13). Sucrose phosphorylase (E.C.2.4.1.7) is a disaccharide glucosyl transferase the presence of which in developing grain has not been reported, and as far as is known, is restricted to micro-organisms (Mieyal and Abeles, 1972). Amylosucrase (E.C.2.4.1.4) is also a bacterial enzyme and catalyses the synthesis of an amylopectin-type molecule. This enzyme has not been found in plant extracts (Okada and Hehre, 1974). No doubt any reports of low activity in plant materials would uncharitably be attributed to microbial contamination!

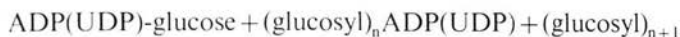
In sweet corn (de Fekete and Cardini, 1964), barley (Baxter and Duffus, 1973a) and rice (Perez *et al.*, 1975) it is considered that the UDP-dependent sucrose synthase probably plays a major role in the sucrose-starch conversion. The evidence is based largely on the high relative activity of the UDP-dependent enzyme throughout grain development, as compared to both the ADP-dependent enzyme and soluble invertase (E.C.3.2.1.26). When making such conclusions certain unwarrantable assumptions are often made. One is that the same fraction of total enzyme activity is extracted at each stage of development; a second is that enzymes are not subject to differential activation or inhibition during extraction; and a third assumption is that *in vitro* activities are related to those *in vivo*. While experimentation is almost impossible in the absence of such assumptions they should be kept in mind in the course of the following discussion. Comparisons are frequently made difficult because for example, the rice results shown above were based on measurements with intact grains which presumably contained the embryo and outer layers whereas the barley results were obtained with isolated endosperms. When the developing barley embryo is considered separately the mechanism of sucrose utilization appears to be slightly different from that of the endosperm (Duffus and Rosie, 1975). While significant activities of ADP- and UDP-dependent sucrose synthase could be detected only after 21–25 days after anthesis, invertase activity was present in significant amounts as early as 18 days after anthesis. Thus it is possible that in the very early stages some sucrose is cleaved by invertase during entry and diffuses as monosaccharides to the embryo cells. By 25 days the system shifts to a pattern similar to that in the barley or rice endosperm. In maize Tsai *et al.* (1970) have shown a peak of invertase activity at 12 days after pollination, before any detectable sucrose synthase. Thus it may be that initially sucrose is hydrolysed to free monosaccharides and could be resynthesized later in cells active in starch synthesis, or converted directly to nucleotide sugars via glucose-1-phosphate. It was considered that the high invertase activity of the pedicel and/or placentochalazal tissues was responsible for the cleavage (Shannon and Dougherty, 1972). In wheat (Sakri and Shannon, 1975) when  $^{14}\text{C}$ -fructosyl sucrose was injected into the peduncle there was little randomization of the  $^{14}\text{C}$  between the fructose and glucose moieties of sucrose extracted from pericarp and endosperm, compared to that in the rachis. The most obvious

explanation is that the sucrose moves out of the phloem and into the endosperm cells without hydrolysis. There may then be some differences between the initial stages of development in maize grain and those in barley, rice and perhaps wheat, since in the former the precursors of starch synthesis are the free sugars glucose and fructose and in the latter they are presumably UDP-glucose and fructose.

#### B. NUCLEOTIDE SUGAR METABOLISM

The enzymes involved in the transformation of hexose to glucose-1-phosphate-hexokinase (E.C.2.7.1.1), glucose-6-phosphate ketoisomerase (E.C.5.3.1.9) and phosphoglucomutase (E.C.2.7.5.1) (de Fekete, 1969), are present in both immature barley endosperm (Baxter and Duffus, 1973a) and the rice grain (Perez *et al.*, 1975) soon after anthesis. The further metabolism of glucose and fructose in immature maize endosperm to glucose-1-phosphate is probably also via the same pathway (Tsai *et al.*, 1970). The glucose-1-phosphate is then presumably converted to nucleotide sugars and thence to starch.

The fate of the UDP-glucose formed as a result of UDP-dependent sucrose synthase is not completely resolved since, as we shall see, the nucleotide sugar donor for the starch synthase (E.C.2.4.1.21) of developing rice (Murata *et al.*, 1963), maize (Recondo and Leloir, 1961) and barley (Baxter and Duffus, 1973b) is considered to be ATP-glucose rather than UDP-glucose:



Murata *et al.*, (1966) could not detect any transglucosylation reaction which might convert UDP-glucose to ADP-glucose in immature rice grains. de Fekete and Cardini (1964), using immature sweet corn, have suggested that the UDP-glucose produced as a result of sucrose synthase activity may be converted to ADP-glucose, first by conversion to glucose-1-phosphate via UDP-glucose pyrophosphorylase (E.C.2.7.7.9) and thence to ADP-glucose by a reversal of ADP-glucose pyrophosphorylase:



Both these enzymes have subsequently been shown to be present in developing wheat (Turner, 1969), maize (Ozbun *et al.*, 1973), barley (Baxter and Duffus, 1973a) and rice (Perez *et al.*, 1975) grains. In barley the UDP-glucose enzyme was detected five days before the ADP-glucose enzyme first appeared. In rice grains they were both active from 1-3 days after flowering. The amount of ADP-glucose pyrophosphorylase in rice, although low in comparison to UDP-glucose pyrophosphorylase was apparently adequate to account for all the starch produced from both the glucose and fructose moieties of sucrose (Perez *et al.*, 1975), assuming of course that starch turnover was minimal.

It has been suggested that the ADP-glucose pyrophosphorylases have a regulatory role in starch synthesis (Preiss and Levi, in press). However, the enzyme from maize endosperm, since it is comparatively insensitive to activation and inhibition by 3-phosphoglycerate (Dickinson and Preiss, 1969) and phosphate (Amir and Cherry, 1972), was thought to demonstrate that this tissue does not require allosteric regulation (Preiss and Levi, in press). On the other hand this may only mean that starch synthesis in endosperm is regulated at a site other than that of ADP-glucose pyrophosphorylase. However, Amir and Cherry (1972) have shown that pyrophosphate is a potent inhibitor of ADP-glucose formation in the reaction catalysed by ADP-glucose pyrophosphorylase from immature sweet corn endosperm. They have suggested that the equilibrium of this reaction could be regulated by pyrophosphate concentration. This in turn is controlled by the enzyme inorganic pyrophosphatase (E.C.3.6.1.1) which is low in activity throughout the first 18 days of barley endosperm development but reaches a maximum value at 25 days after anthesis (Baxter and Duffus, 1973a).

Thus at early stages the equilibrium will favour glucose-1-phosphate formation from UDP-glucose and at later stages the increase in pyrophosphatase activity would allow synthesis of ADP-glucose by a reversal of the equilibrium. If indeed the pyrophosphate inhibition is selective then a coupling of UDP-glucose pyrophosphorylase in the direction of glucose-1-phosphate synthesis, with ADP-glucose pyrophosphorylase in the direction of ADP-glucose synthesis, would ensure a rapid conversion of sucrose to starch (Fig. 7). Other enzymic mechanisms possibly involved in the sucrose-starch conversion are discussed by Murata *et al.* (1966).

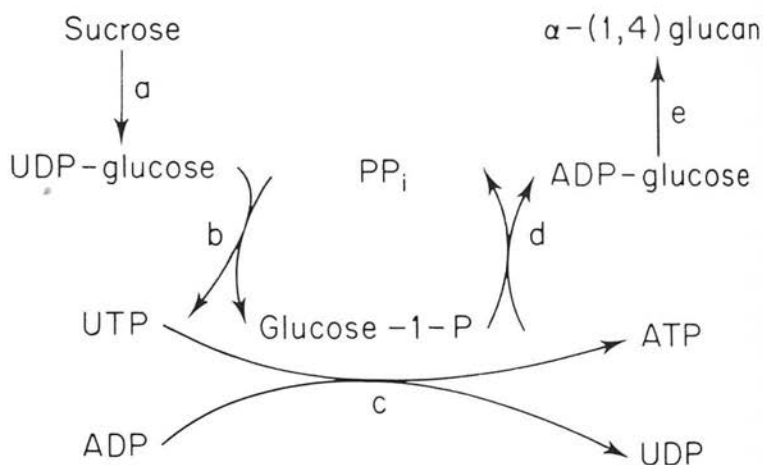


FIG. 7. Suggested mechanism for conversion of sucrose to  $\alpha$ -(1,4) glucan via UDP-glucose and ADP-glucose.

a, UDP-dependent sucrose synthase; b, UDP-glucose pyrophosphorylase; c, nucleoside diphosphate kinase; d, ADP-glucose pyrophosphorylase; e, starch synthase.

Nucleoside diphosphate kinase (E.C.2.7.4.6), which is present in both immature barley (Baxter and Duffus, 1973a) and rice grain (Perez *et al.*, 1975) will enable the UTP formed from UDP glucose (via UDP glucose pyrophosphorylase) to be re-utilized as ATP which is then used in ADP-glucose biosynthesis. Changes in activity of a number of enzymes concerned in the conversion of sucrose to sugar nucleotides are shown in Table III. Thus a mechanism is available (Fig. 7) whereby UDP-glucose, initially formed from sucrose, can be utilized in the biosynthesis of ADP-glucose.

### C. STARCH BIOSYNTHESIS

#### 1. Normal Development

Starch structure and general metabolism have been recently reviewed by Manners (1974). Preiss and Levi have additionally reviewed starch metabolism in leaves. The discussion which follows complements these reviews and is restricted to those aspects of particular relevance to grain development.

Traditionally starch phosphorylase (E.C.2.4.1.1) was considered to be the enzyme responsible for the synthesis of the  $\alpha$ -(1,4) glucosidic linkage:



However, in 1961 Leloir *et al.* showed that the synthesis of starch could be catalysed by starch synthase. Recondo and Leloir (1961) showed that ADP-glucose rather than UDP-glucose was the preferred substrate. Preiss and Levi consider that starch phosphorylase is not functional in the biosynthetic pathway largely on the basis of a possibly unfavourable  $\text{P}_i$ /glucose-1-phosphate ratio and also because the reaction catalysed by starch phosphorylase is reversible where the starch synthase reaction is irreversible in the direction of  $\alpha$ -(1,4) glucan synthesis. However, accurate measurements of intracellular concentrations of glucose-1-phosphate and  $\text{P}_i$  (or any other metabolites) have not been made during endosperm development nor is it yet possible to do so in the vicinity of the enzyme. Thus, a possible synthetic role for starch phosphorylase cannot be disregarded. In particular the question as to whether a starch synthase exists which can function without a primer remains unresolved (Preiss and Levi, in press). A number of workers (Tsai and Nelson, 1969; Baxter and Duffus, 1973c; Burr and Nelson, 1973; Perez *et al.*, 1975) have reported phosphorylase isoenzymes in developing grains which do not require a primer. These observations became of particular importance when it was reported that starch synthase activity was not measurable during the initial stages of development in endosperms of barley (Baxter and Duffus, 1971) but ample phosphorylase activity could be demonstrated. Additionally, starch granules were observed to be present although starch synthase activity was not. However, it is easier to report the absence of enzyme activity than its

TABLE III

Changes in activities of enzymes involved in metabolism of sucrose, glucose-1-phosphate and nucleotide sugars in developing IR 1541-76-3 rice grains

Enzyme activity/min/grain	Days after flowering						LSD <sup>a</sup> (5%)
	1-3	5-6	8-9	11-12	17-18	21	
Bound invertase (nmol sucrose)	2.2	4.5	12.5	15.2	19.8	1.8	3.3
Soluble invertase (nmol sucrose)	4.3	6.1	10.2	6.4	3.6	1.2	2.3
UDP-dependent sucrose synthase (nmol sucrose)	2.8	28	37.5	42.8	32	6.9	11.4
ADP-dependent sucrose synthase (nmol sucrose)	trace	2.3	4.5	7.2	1.4	1.6	4.2
Hexokinase (nmol NADPH)	0.8	5.5	12.4	15.8	8	0.9	2.1
Phosphoglucosmutase (nmol Pi)	5	10.7	32.2	49.4	80.1	4.1	14.9
UDP-glucose pyrophosphorylase (nmol UDP-glucose)	29.6	45.7	62.8	93.1	64.7	68	24.4
ADP-glucose pyrophosphorylase (nmol ADP-glucose)	6.3	9.2	11.7	12.2	11.6	14.6	n.s.
Nucleoside diphosphate kinase (nmol ATP)	8.7	9.9	8.5	6.8	7	9.9	1

<sup>a</sup>Least significant difference (Data from Perez *et al.*, 1975).



presence and certainly Ozbun *et al.* (1973) in maize and Perez *et al.* (1975) in rice, using more sensitive methods, were able to detect significant activity of ADP-glucose starch synthase in developing kernels at 1–3 days after flowering and 8 days post-pollination respectively. It is probable, however, that while phosphorylase may well be involved in the initiation of starch synthesis, the bulk of  $\alpha$ -(1,4) glucan synthesis in developing cereal grain is catalysed by starch synthase.

Starch synthases are present either bound to the starch granule or soluble in the cytoplasm. If all endosperm starch is derived from a reaction catalysed by starch synthase, then during the initial stages of development the enzyme may exist in the soluble form. Certainly in barley (Baxter and Duffus, 1973b) UDP-glucose starch synthase is initially predominantly soluble, becoming progressively bound as the tissue matures. In rice (Perez *et al.*, 1975) the ratio of soluble to bound starch synthase activity with ADP-glucose is greatest 1–3 days after flowering, and least 21 days after flowering. Again, as development proceeds the enzyme becomes increasingly amyloplast-bound; indeed the soluble primed forms of phosphorylase and ADP-glucose starch synthase were the only soluble enzymes capable of synthesizing starch that had appreciable activities throughout the period of starch accumulation. In general it appears that the soluble enzyme prefers ADP-glucose, although in barley at 20 days after anthesis, UDP-glucose is the preferred sugar nucleotide.

The relative contributions of the soluble and amyloplast-bound starch synthases to starch deposition is unclear. Ozbun *et al.* (1973) have presented results which suggested that, over the period 8–28 days after pollination in *normal*, *waxy*, *amylose-extender* and *shrunk-4* maize, less than 10% of total activity was granule bound. However, the ADP-glucose concentration used for measurement of granule-bound enzyme may have been limiting so that maximum rates were not obtained. Furthermore, maximum activities may not be achieved when the enzyme is complexed intimately with the starch granule. Certainly mechanical disruption (Frydman and Cardini, 1967) of the starch granule increases activity of the bound enzyme. The amyloplast-bound starch synthases from barley (Baxter and Duffus, 1971) and rice (Perez *et al.*, 1975) use ADP-glucose more efficiently than UDP-glucose, although in barley amyloplast-bound starch synthase activity was initially greater with UDP-glucose. The presence of amyloplasts of different sizes and compositions in immature endosperms (see Section III) suggests that there may be several amyloplast-bound starch synthases. The starch granule-bound starch synthase of rice (Murata *et al.*, 1964; Perdon *et al.*, 1975) and barley (Baxter and Duffus, 1971) can use both nucleosides. These findings may be accounted for in part by the finding (Williams and Duffus, 1977) that the nucleoside specificity of the starch synthase associated with each population of amyloplasts varies independently during development (Fig. 8). Changes in activity of starch synthases in developing rice grains are shown in Table IV.

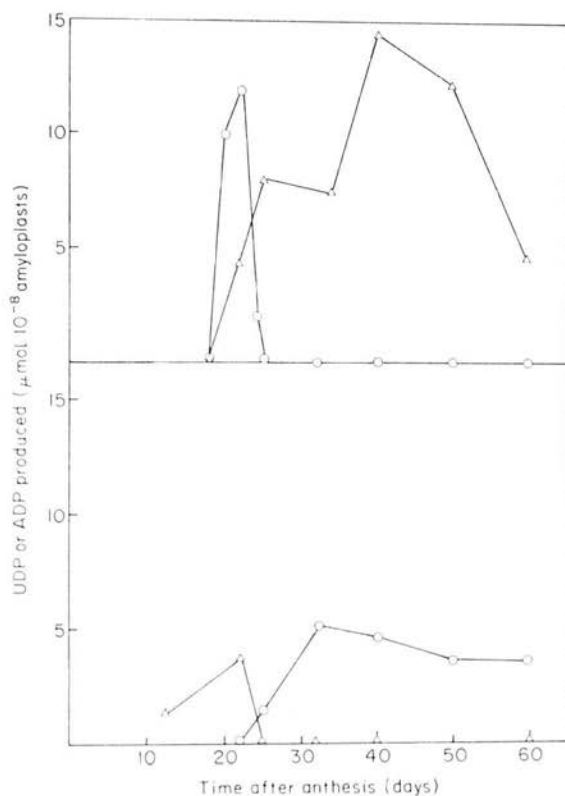


FIG. 8. Starch synthase activity in large and small amyloplasts throughout development in barley endosperm. A, starch synthase activity in large amyloplasts; B, starch synthase activity in small amyloplasts.  $\circ$ , activity with UDPG;  $\triangle$ , activity with ADPG. Mean SD =  $\pm 2.2$  (Williams and Duffus, 1977).

Starch synthase, as described above, catalyses the synthesis of  $\alpha$ -(1,4) glucans and an additional enzyme, called branching enzyme (E.C.2.4.1.18) or Q-enzyme, introduces  $\alpha$ -(1,6) linkages into the straight chain amylose by transfer of a short chain of  $\alpha$ -(1,4) linked glucose residues to form amylopectin (Fig. 9).

Amylopectin accounts for some 80–90% of the starch initially formed in developing maize, wheat and barley endosperms (see Section III) and decreases in normal grain to around 75% of total starch. Since the assay of branching enzyme is made particularly difficult in immature cereal endosperms due to the presence of interfering amylases, little is known of its relationship to starch synthases and in particular, how the tissue produces its constant proportions of the two polymers, amylose and amylopectin. A soluble branching enzyme is present in immature barley endosperm (Williams, 1976), and Boyer and Preiss (1977) have shown the presence of multiple forms of soluble branching enzyme in immature maize endosperm.

TABLE IV  
Changes in activity of starch synthases in developing IR1541-76-3 rice grains

Enzyme activity/min/grain	Days after flowering					LSD <sup>a</sup> (S%)
	1-3	5-6	8-9	11-12	17-18	21
Soluble primed ADP-glucose starch synthase (nmol glucose)	0.42	2.48	3.85	2	2.02	2.25
Soluble primed UDP-glucose starch synthase (nmol glucose)	0.03	0.43	0.31	0.35	0.35	0.39
Bound ADP-glucose starch synthase (nmol glucose)	trace	0.36	1.47	3.20	4.98	9.72
Bound UDP-glucose starch synthase (nmol glucose)	0.002	0.02	0.05	0.12	0.16	0.22

<sup>a</sup> Least significant difference (Data from Perez *et al.*, 1975).

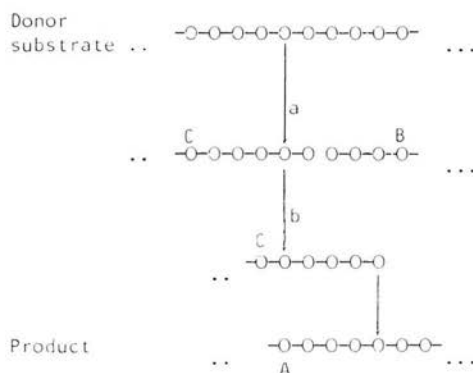


FIG. 9. Formation of an  $\alpha(1,6)$ -glucosidic inter-chain linkage by Q-enzyme (branching enzyme). A short chain of glucose residues (C) is transferred from a donor substrate to an acceptor (A) which may be the remaining part of the original chain (B) (i.e. intra-chain transfer) or an adjacent chain (i.e. inter-chain transfer).  $\text{O---}$ ,  $\alpha(1\rightarrow4)$ -linked D-glucose residue;  $\downarrow$ ,  $\alpha(1\rightarrow6)$  inter-chain linkage; a, scission of a non-terminal  $\alpha(1\rightarrow4)$  linkage; b, formation of an  $\alpha(1\rightarrow6)$  linkage (Manners, 1974).

Two forms—I and II—were distinguished on the basis of their behaviour in the assay systems used. Branching enzyme II was additionally separated into two fractions IIa and IIb where IIa had less activity with amylopectin than IIb (Boyer and Preiss, 1978).

## 2. Mutant Endosperms

Most research in this area has been with maize, and cereals such as barley and wheat have been relatively neglected. The subject has recently been reviewed, with regard to maize, by Nelson (1975).

A number of loci are known in maize at which mutations affect the quality of starch synthesized (see Section II). From consideration of the effect of these mutations on starch synthesis Nelson (1975) has tentatively concluded that there are probably more enzyme systems participating in starch synthesis in cereal endosperm than most biochemists imagine. It has been observed that mutants of maize (Fuwa, 1957) and rice (Baun *et al.*, 1970) differing in amylose content contained similar levels of soluble branching enzyme activity. Thus it may be that the extent of branching is not determined by branching enzyme only. On the other hand waxy rice grains (Baun *et al.*, 1970) contained much lower levels of bound starch synthase than non-waxy grains. It was suggested that formation of a complex between newly synthesized amylose and bound starch synthase may reduce access to branching enzyme and hence lower the proportion of amylopectin formed as development proceeds. In the absence of bound synthase all amylose, postulated in developing waxy maize to be derived from soluble ADP-glucose starch synthase (Ozbun *et al.*, 1971), would be open to attack from branching enzyme resulting in a highly

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FIG. 10  
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A scheme showing a number of the possible reactions whereby sucrose may be converted to starch in developing endosperms is shown in Fig. 10 (Perez *et al.*, 1975).

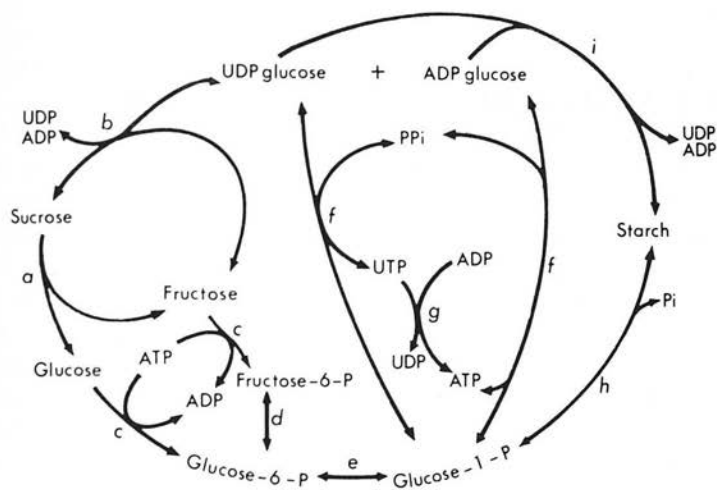


Fig. 10. Scheme showing a number of reactions which may be involved in the conversion of sucrose to starch in developing endosperms. a, invertase; b, UDP(ADP)-dependent sucrose synthase; c, hexokinase; d, glucose-6-phosphate ketoisomerase; e, phosphoglucumutase; f, UDP(ADP)-glucose pyrophosphorylase; g, nucleoside diphosphate kinase; h, phosphorylase; i, ADP(UDP)-glucose starch synthase (Perez *et al.*, 1975).

## V. STARCH DEGRADATION DURING GRAIN DEVELOPMENT

Development in cereal grains is characterized by a net synthesis and deposition of starch. Simultaneously, however, catabolic processes are known to be present which are active in starch hydrolysis and degradation. This is not really surprising, since energy is obviously required for the great variety of synthetic processes carried out by the endosperm cells during development.

Both glycolytic (Duffus and Rosie, 1977) and mitochondrial enzyme (Duffus, 1970) activities are present in developing barley endosperm. This, together with the presence of mitochondria (Williams and Duffus, 1978) in association with the amyloplasts (Section II), suggests that active respiratory processes accompany grain development and may supply energy for starch and protein synthesis.

It is not clear whether incoming sucrose or granular starch itself is the direct substrate for energy production. Certainly sucrose could be diverted to glycolysis after conversion to glucose-1-phosphate as described previously (Section IV A). On the other hand, a number of starch hydrolysing enzymes are known to be present in immature grains of barley (Duffus and Rosie, 1973a), wheat and oats (Meredith and Jenkins, 1973) and triticale and rye (Jenkins and Meredith, 1975). They may be involved in the production of energy from starch or possibly in the synthesis of primers for the starch synthase reaction.

$\alpha$ - (E.C.3.2.1.1) and  $\beta$ -amylases (E.C.3.2.1.2) of developing barley grains have been of particular interest to barley breeders in their search for genotypes with enhanced amylolytic properties of value to maltsters. They are also of interest since their presence may affect the starch composition (Allison *et al.*, 1974) and content of the mature barley grain. However, Riggs and Gothard (1976) in a study of  $\alpha$ -amylase activity in seven spring barley cultivars during grain maturation, found no relationship between grain growth rate and  $\alpha$ -amylase activity (Fig. 11). However,  $\alpha$ -amylase activity is very low in comparison to total hydrolytic activity (Duffus and Rosie, 1973a), and a relationship might more easily be sought between total starch hydrolysing capacity and yield of starch. MacGregor *et al.* (1972) have suggested that the disappearance of the pericarp starch granules (Section II A), which occurs early in grain development, may be a result of  $\alpha$ -amylase activity. It was further suggested that the pericarp may act as a temporary energy store for the growing kernel. Allison *et al.* (1974) have also suggested that the pericarp may also act as a source of primer to initiate starch synthesis in the very young endosperm.

$\beta$ -Amylases are present in high activity in developing barley grains and a proportion of these are converted during maturation to a bound form (LaBerge *et al.*, 1971). Again, this enzyme may have similar functions to those suggested for  $\alpha$ -amylase. Preliminary studies (Allison and Ellis, 1973) on the

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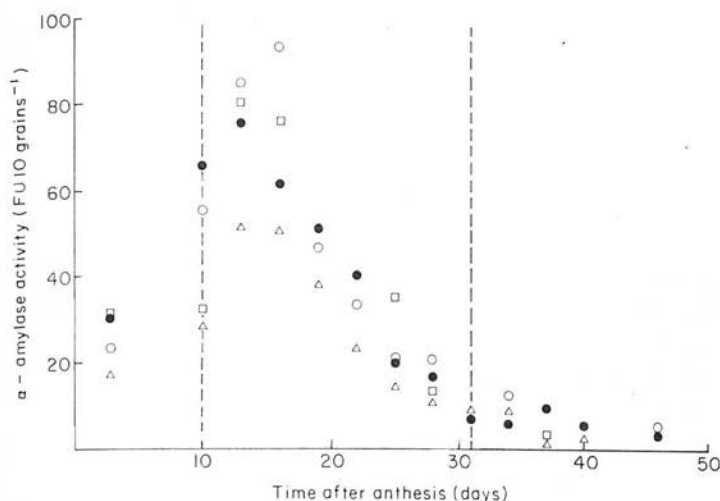


FIG. 11. Total  $\alpha$ -amylase activity in Farrand units (FU) per 10 grains during grain development in barley. ●, Maris Mink; ○, Maris Dingo; △, Glacier; □, Proctor. Between 10 and 31 days after anthesis there was a substantially linear phase of growth (Riggs and Gothard, 1976).

distribution of  $\beta$ -amylase zymotypes in developing grain have indicated that this polymorphism is related to a  $\beta$ -amylase polymorphism in barley malt. Again therefore, these observations may be helpful in early forecasting for high diastase barleys. Degradative phosphorylase activity (Duffus and Rosie, 1973a) is also present in immature barley grain, but the activity is rather less than that of  $\alpha$ -amylase.

Amylase activity in immature wheat grains has been subject to particular investigation for a number of reasons: firstly because of the adverse effect of  $\alpha$ -amylase on the baking quality of wheat flours (Irvine, 1975); secondly because of the possible relationship between  $\alpha$ -amylase of immature wheat grains and premature sprouting; and thirdly, because the shrivelling of frost-damaged wheat grain appears to be associated with high amylase activity (Meredith, 1977).

Starch degradation has been discussed in some detail by Manners (1974). It is generally accepted that an  $\alpha$ -amylolytic breakdown of starch represents the major catabolic pathway. The resulting dextrins may then be degraded by  $\beta$ -amylase, limit dextrinase (E.C.3.2.1.10) and  $\alpha$ -glucosidase (3.2.1.20) (Dunn, 1974). The latter two enzymes are present during development in barley grains (Williams, 1976). However, the low activity of  $\alpha$ -amylase in endosperm, coupled with the observation that extracts from germinated barley do not degrade immature amyloplasts (Williams and Duffus, 1977), suggests that soluble dextrins or sucrose rather than intact starch granules are the source of energy for grain development. The role of enzymes of starch degradation in

developing grain can only be satisfactorily resolved when mutants without the enzymes are located or when some means of selective inhibition is devised.

## VI. ROLE OF THE TESTA-PERICARP AND SURROUNDING TISSUES DURING GRAIN DEVELOPMENT

Cereal grains such as wheat, oats and rye which have chlorophyll-containing surrounding tissues such as the testa-pericarp, glumes and paleae (Section II) have the ability to carry out photosynthesis. Carr and Wardlaw (1965) have shown that all the separated parts of the immature wheat ear including outer glumes, lemmas, paleae, rachis and intact grains, are capable of light-dependent carbon dioxide fixation. Up to 80% of the carbon assimilated by the glumes moved into the grains.

Photosynthesis by the grain itself takes place in the green layer of the pericarp. For much of the developmental period this is a bright emerald-green tissue surrounded by a transparent outer layer. In both barley (Duffus and Rosie, 1973b) and wheat (Wirth *et al.*, 1977) the pericarp has been shown to be capable of photosynthesis and contains a number of enzymes normally associated with photosynthetic carbon dioxide fixation. Of particular interest was the high activity, relative to the leaves, of phosphoenol pyruvate carboxylase (E.C.4.1.1.31) in pericarps of barley, wheat and oats. The additional presence of pyruvate orthophosphate dikinase (E.C.2.7.9.1) in barley (Duffus and Rosie, 1973b) and wheat (Wirth *et al.*, 1977) pericarps, an enzyme which, with high activities of phosphoenol pyruvate carboxylase, is characteristic of  $C_4$  rather than  $C_3$  photosynthesis, was of particular interest. On the basis of leaf photosynthesis both barley and wheat are considered to be  $C_3$  plants. The first-formed product of photosynthesis in immature barley pericarp is the  $C_4$  acid malate which is then rapidly converted to sucrose (Nutbeam and Duffus, 1976). Evidence suggests that this sucrose may be transported to the endosperm where it is incorporated into starch (A. R. Nutbeam, in preparation). However, an investigation of the outer epidermis of the barley pericarp using scanning electron microscopy has revealed very few stomata. Examination of these cells by transmission electron microscopy (Fig. 3) has demonstrated that they have a distinct cuticle on their outer surface (M. P. Cochrane, in preparation), and in addition Radley (1976) has shown that diffusion of atmospheric carbon dioxide into the pericarp is severely limited. It is therefore likely that the fixed carbon dioxide may be derived from endosperm respiratory processes rather than the outer atmosphere. Similarly it is possible that oxygen evolved from photosynthesis may remain inside. It may then be that the presence or absence of oxygen may regulate endosperm respiratory processes and hence the supply of carbon dioxide for fixation by the pericarp. Thus the pericarp, in addition to its

function as a translocatory tissue, may be involved in the control of the entire metabolic activity of the endosperm.

## VII. CONCLUSIONS

It is clear that the developing cereal grain is far from being an inert storage tissue. It is active in synthesizing a wide range of metabolites and precursors as well as the more obvious macromolecules such as starch and protein. Simultaneously, the period of deposition of storage materials is associated with active catabolic processes which, far from being wasteful, may be required for energy production, primer synthesis and valuable intermediates for example, the keto acids required for protein synthesis.

The physiological and biochemical factors limiting grain yield and controlling composition are still a matter for conjecture. For example, it is still a matter of speculation whether grain yield is limited by photosynthesis or by the regulatory systems within the grain itself (Yoshida, 1972). Jenner and Rathjen (1977) have suggested that in wheat the decline in accumulation of starch is caused by a fall in synthetic capacity of the endosperm and not by a reduction in the supply of sucrose. Further work should be aimed at identifying the sites of regulation within the grain and discovering how their effects may be modified. Some of these sites may involve the diversion of intermediates from the pathways of carbohydrate degradation into protein synthesis. Whether such diversion is desirable should be considered.

Finally, the biochemical mechanism whereby starch composition is so closely regulated during development is little understood. It is clear, however, that while the properties and composition of the amyloplasts and their contents no doubt affect their ease of hydrolysis in malting or in digestion by animals, the precise reasons are largely unknown. The regulation of starch synthesis and its subsequent utilization may thus continue to occupy plant physiologist and biochemists for some considerable time.

## ACKNOWLEDGEMENTS

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## The Nucellar Projection and Modified Aleurone in the Crease Region of Developing Caryopses of Barley (*Hordeum vulgare* L. var. *distichum*)

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### Summary

A description is given of morphological changes which occur in the nucellar projection and crease aleurone cells of barley caryopses between 10 and 35 days after anthesis. Transfer cells are found in the nucellar projection at all stages. Some cells of the nucellar projection bordering the endosperm cavity have stacked cisternae of endoplasmic reticulum. The massive wall thickening observed in young cells of crease aleurone and in some cells of the nucellar projection appears to be composed of callose. The endomembrane system of the young crease aleurone cells is extensive and complex. Staining with alcian blue indicates that mucopolysaccharide is present in the endosperm cavity. The significance of the structures observed and the presence of callose and mucopolysaccharide is discussed in relation to the movement of substances across the crease region into the developing endosperm.

**Keywords:** Barley; Callose; Crease aleurone; Endoplasmic reticulum; Nucellar projection; Transfer cells.

### 1. Introduction

The nucellar projection and the modified aleurone of the crease region of the barley caryopsis have different ontogenetic origins but they have been investigated together because they lie on either side of a gap in the route along which nutrients move from the vascular tissue of the pericarp to the developing endosperm and embryo (FRAZIER and APPALANAIDU 1965).

One of the many situations in which transfer cells have been found consistently is "in association with the cytoplasmic discontinuities and nutritional relationships existing between the alternating generations of plants" (GUNNING and PATE 1969). ZEE and O'BRIEN (1971) reported the presence of wall ingrowths

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in the nucellar projection of *Echinochloa utilis* and HUGHES (1976) observed flange-type transfer cells in the nucellar projection of wheat but otherwise in cereals, both the structure and function of this tissue have virtually been ignored.

In contrast, considerable attention has been paid to the outermost endosperm cells in the placental region of *Gramineae* caryopses. These are continuous with the cells which make up the aleurone layer and are known as modified aleurone, crease aleurone or groove aleurone even though they may contain few aleurone grains at maturity and none at all during the first two to three weeks of development (BRADBURY *et al.* 1956, FULCHER *et al.* 1972, MORRISON *et al.* 1978). EVERS (1970) suggested that in wheat the thick-walled aleurone cells of the crease play a significant part in determining the shape of the mature grain but most other workers have concentrated on their possible role in solute transfer.

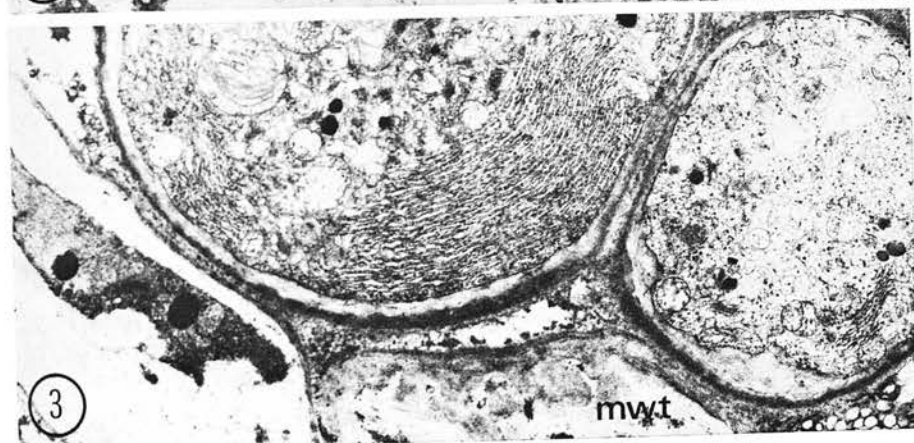
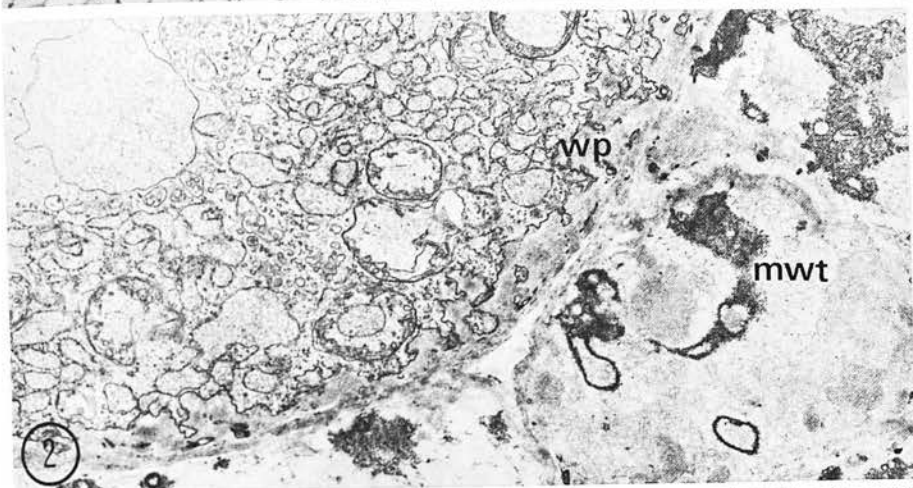
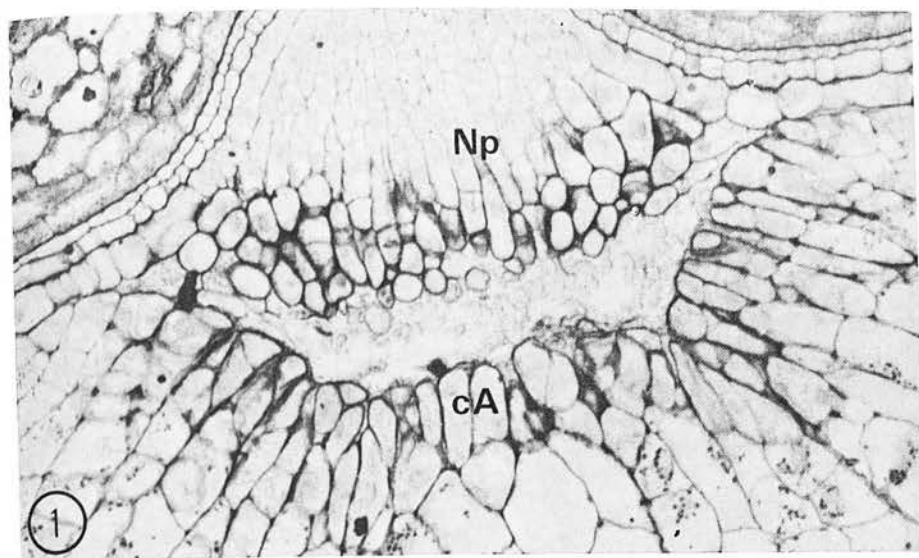
Transfer aleurone cells have been found in the hilum region of *Echinochloa utilis* (ZEE and O'BRIEN 1971), *Setaria lutescens* (ROST and LERSTEN 1971), *Sorghum bicolor* (GILES *et al.* 1975) and *Paspalum dilatatum* (IPAGUIRRE-ARTUCIO and ZILIANI 1975) but ZEE and O'BRIEN (1971) and BECHTEL and POMERANZ (1977) did not detect any transfer aleurone cells in the grooves of wheat and rice respectively. MORRISON *et al.* (1978) investigated the groove aleurone in young caryopses of wheat and found that soon after these cells are formed their walls acquire massive and irregular thickening and, as their cytoplasm contains distended cisternae of endoplasmic reticulum and relatively few mitochondria, these workers did not consider them to be transfer cells. However, AYRE and ANGOLD (1979) published a micrograph of a crease aleurone cell of wheat and reported that the characteristic wall ingrowths of the transfer cell appeared in the aleurone cells themselves 15 days after anthesis.

Most, if not all, of the material ultimately stored in the endosperm of cereals is thought to pass through the tissues of the crease. Thus the purpose of this investigation was to trace the development of these tissues during the grain-filling period in barley and to try to relate the features observed to the processes of solute transfer and endosperm growth.

Fig. 1. Nucellar projection (*Np*) and crease aleurone (*ca*) of a 10-day-caryopsis of greenhouse-grown barley cv. Julia. Toluidine blue staining.  $\times 180$

Fig. 2. The nucellar projection bordering the endosperm cavity of a 10-day-caryopsis of greenhouse-grown barley cv. Julia. Adjoining cells with massive wall thickening (*mwt*) is a cell with wall protuberances (*wp*) and extensive tubular cisternae of endoplasmic reticulum.  $\times 10,000$

Fig. 3. The nucellar projection bordering the endosperm cavity of a 12-day-caryopsis of field-grown barley cv. Midas. Some cells have massive wall thickening (*mwt*) and outside these are cells containing stacked cisternae of endoplasmic reticulum.  $\times 6,500$



Figs. 1-3

## 2. Materials and Methods

### 2.1. Growth of Plants

Barley plants (*Hordeum vulgare* L. var. *distichum* cultivars Midas and Julia) were grown in a greenhouse. The conditions of growth and the methods used to determine the developmental age of caryopses were the same as those described previously (COCHRANE and DUFFUS 1979). Additional material was examined from plants grown in the field, and in a growth chamber in which the temperature was 10 °C at night and 11.5 °C during a 16-hour-day when the illumination was approximately 440  $\mu\text{Einsteins m}^{-2} \text{sec}^{-1}$  at pot level.

### 2.2. Electron Microscopy

Transverse slices 1 mm thick were cut from the middle of caryopses under a fixative containing 2.5% w/v glutaraldehyde in 0.025 M phosphate buffer pH 7.2. The tissue slices were kept in this fixative at 4 °C for 20 hours, post-fixed for 2 hours in 2%  $\text{OsO}_4$  at room temperature, dehydrated in an acetone series and embedded in an epon/araldite resin. Thin sections were stained in uranyl acetate and lead citrate and examined in an AEI EM 802 electron microscope at 60 kV.

### 2.3. Light Microscopy

Sections cut from blocks prepared for electron microscopy were stained in toluidine blue. Free-hand sections of fresh caryopses were immersed in a solution of 1% alcian blue in 3% acetic acid for 10 s. The stain was removed by blotting and the sections mounted in a glycerol/gelatin mixture containing phenol. (LEV and SPICER 1964).

### 2.4. Aniline Blue Induced Fluorescence

The dyes used were (1) Aniline blue C.I. No. 42755 Batch 3561 supplied by Raymond Lamb Ltd. (2) Soluble blue 24085. (3) Soluble acid blue R and (4) Soluble blue 22684. W. S. Simpson and Co. (The British Aniline Dye and Chemical Works) Ltd., supplied (2), (3) and (4). The dyes were used in a concentration of 0.05% w/v in 0.067 M phosphate buffer pH 8.5. Free-hand transverse sections of fresh caryopses were mounted in a drop of aniline blue and viewed in a Leitz Ortholux fluorescence microscope using incident light (barrier filter UG 1; exciter filter BG 12). Sections mounted only in buffer and sections stained for 2 minutes in 0.05% w/v aqueous toluidine blue and washed in distilled water before mounting in aniline blue, were also examined (SMITH and MCCULLY 1978).

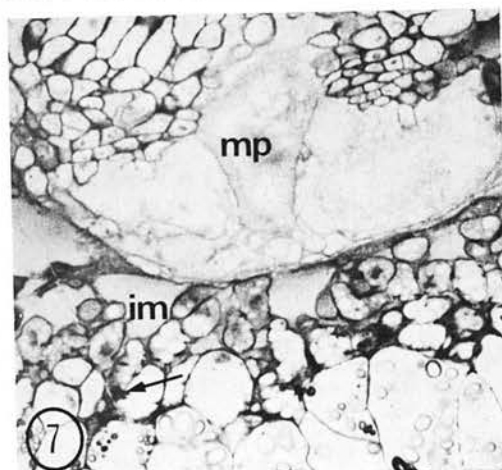
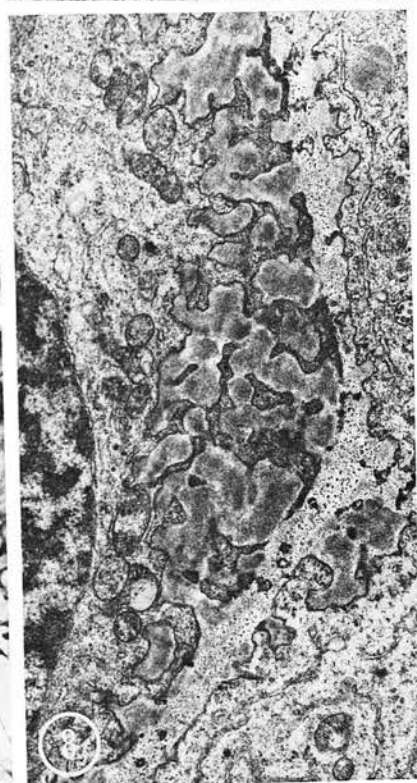
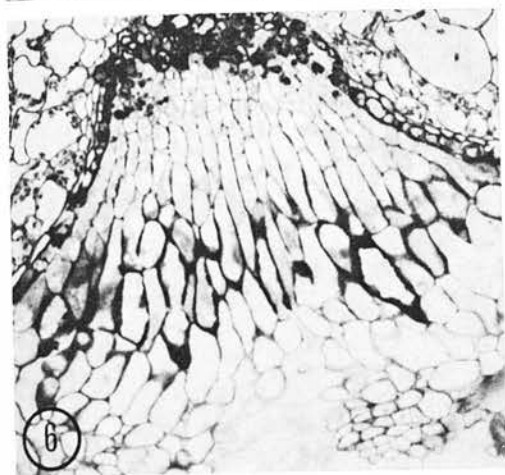
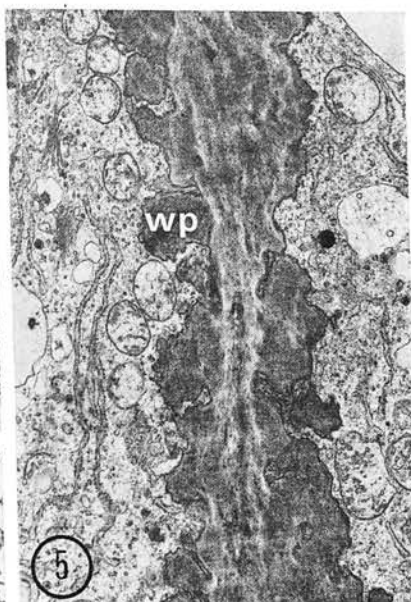
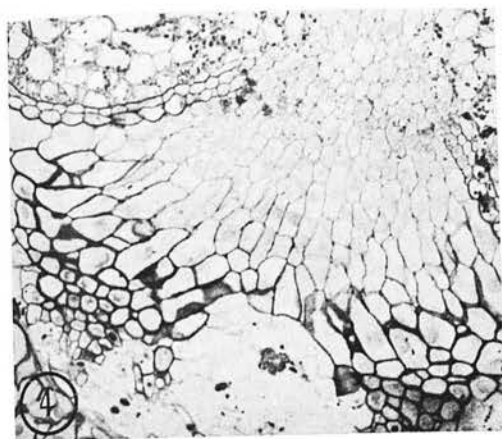
Fig. 4. The nucellar projection of this 20-day-caryopsis of greenhouse-grown barley cv. Midas is divided into two lobes. Toluidine blue staining.  $\times 130$

Fig. 5. Protuberances (*wp*) on the radial wall of a cell in the nucellar projection of a 20-day-caryopsis from greenhouse-grown barley cv. Midas.  $\times 10,500$

Fig. 6. Nucellar projection of a 35-day-caryopsis of barley cv. Midas grown at 10 °C at night, 11.5 °C day. Toluidine blue staining.  $\times 130$

Fig. 7. The endosperm cavity and crease aleurone of the 35-day-caryopsis in Fig. 6. The amorphous material (*mp*) may be mucopolysaccharide. It is different in texture from the homogeneous substance (*im*) separating the crease aleurone cells. Wall projections (arrow) can be seen in the sub-aleurone layers. Toluidine blue staining.  $\times 130$

Fig. 8. Cells in the core of the nucellar projection of a 35-day-caryopsis of greenhouse-grown barley cv. Midas.  $\times 10,500$



Figs. 4-8



### 3. Observations

3.1. The morphological sequences observed were the same in both cultivars and were apparently unaffected by the conditions of plant growth. There were considerable differences in the rates of development of caryopses and so all observations have been related to the developmental time scale (COCHRANE and DUFFUS 1979).

#### 3.2. Nucellar Projection

Seven days after anthesis the barley caryopsis has a well-developed nucellar projection composed of cells which are isodiametric in transverse section. Those bordering the endosperm cavity have slightly thickened walls but all other cells are thin-walled. By 10 days after anthesis the cells of the nucellar projection have elongated considerably in a plane at right angles to the long axis of the caryopsis, two or three cell layers bordering the endosperm cavity have degenerated, and the cells immediately outside them have become thick-walled (Fig. 1). Details of the ultrastructure of nucellar cells from the crease of 10–12-day-caryopses can be seen in Figs. 2 and 3. Some of the intact cells on the periphery of the nucellar projection have wall proliferations characteristic of transfer cells (Fig. 2). However, in other cells the thickening is more massive and is composed of electron-lucent material in which there is a network of more electron-dense material. These wall thickenings often enclose regions of dense cytoplasm.

Many cells near the inner edge of the nucellar projection have a very extensive endoplasmic reticulum (Figs. 2 and 3). In some cells it is in the form of large, mainly rough-surfaced cisternae or tubules, enclosing a granular phase and often protruding into the centre of proplastids. In other cells (Fig. 3) endoplasmic reticulum cisternae are stacked and are swollen only at the ends.

Cells in the core of the nucellar projection have irregular wall protuberances, and contain plastids which have fragments of irregular membrane lattice and several layers of concentric lamellae. These plastids are very similar to those found by DIBOLL (1968) in the chalazal region of the synergids of *Zea mays* L. after fertilization.

As the caryopsis develops the cells of the nucellar projection continue to elongate in the plane at right angles to the long axis of the caryopsis. By 20 days after anthesis they have separated into two lobes, coming closer to the aleurone at the sides but increasing the gap between the tissues at the centre (Fig. 4). The cell walls in the peripheral zone of the nucellar projection are thicker and stain much more densely with toluidine blue than the cell walls in the core of the nucellar projection. However, cells in the core have the flange-like wall protuberances and numerous associated mitochondria which are typical of transfer cells (Fig. 5). The protuberances are more or less homo-



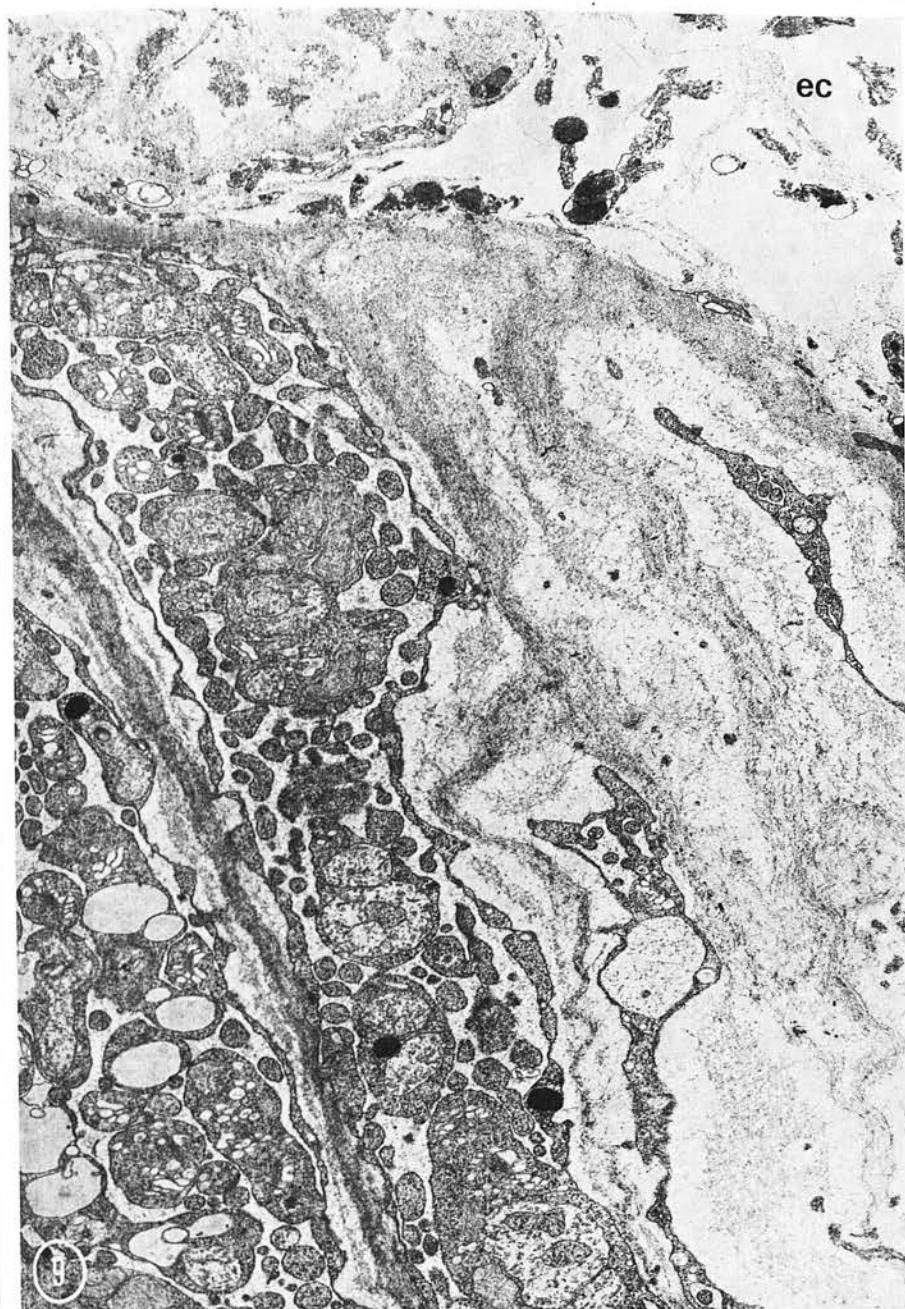


Fig. 9. Aleurone cells bordering the endosperm cavity (*ec*) of a 12-day-caryopsis of field-grown barley cv. Midas. The radial walls are massively thickened with electron-lucent material.  $\times 10,000$

geneous and do not have the outer electron-lucent layer which is frequently observed in transfer cells and is considered to be due to swelling during fixation (BROWNING and GUNNING 1977).

By 30 days after anthesis, the cells of the nucellar projection have ceased to elongate in the plane at right angles to the long axis of the grain. The walls of the band of cells bordering the endosperm cavity no longer stain darkly with toluidine blue but this property is acquired by the walls of the long cells lying next to them (Figs. 6 and 7). Cell walls in the core of the nucellar projection have elaborate wall protuberances and these are observed in caryopses as old as 35 days (Fig. 8). At all ages, wall ingrowths are more extensive on radial than on tangential walls.

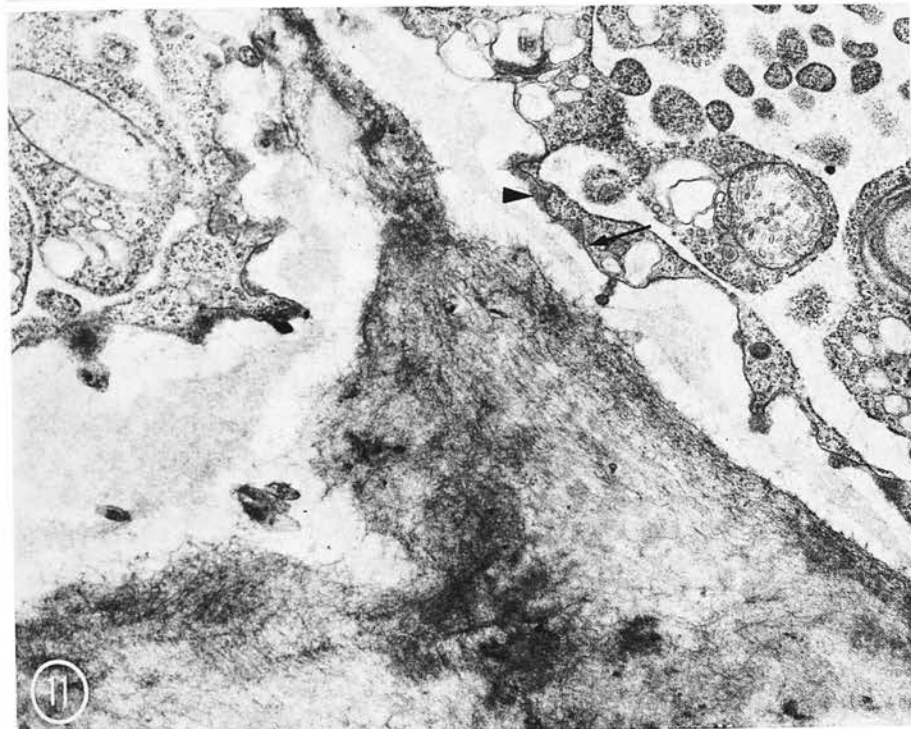
### 3.2. Crease Aleurone

By ten to twelve days after anthesis cell walls have formed throughout the endosperm and at the crease the outer one two cell layers differ from the rest in having dense cytoplasm and no starch grains (Fig. 1). Electron micrographs show that the radial walls of these cells are greatly thickened (Figs. 9 and 10). The thickening material is much less electron-dense than the primary wall and there is reticulate fibrillar material in the centre of each mass of thickening. The thickened cell wall is very irregular in outline and frequently appears to engulf scraps of dense cytoplasm. The cell contents are in two phases, one of which is very dense and is split up into islands of various sizes. These islands contain numerous ribosomes and some contain plastids, mitochondria, vacuoles, and dictyosomes, the last being particularly plentiful. The other phase is comparatively electron-lucent, its staining properties being similar to those of the outer zone of the wall-thickening material. The membrane separating the two phases is frequently seen lying close to the plasma membrane and appears to merge with it in places (Fig. 10).

As caryopsis development proceeds, changes occur in the walls of the crease aleurone cells. Electron-dense material is deposited between the plasma membrane and the electron-lucent layer of wall thickening (Fig. 11). Microtubules have been observed where this third type of wall material is being laid down but were not observed in caryopses fixed during the deposition of the massive electron-lucent wall thickening. Ribosomes are still plentiful in the islands of

Fig. 10. Part of one of the cells in Fig. 9 showing the inclusion of scraps of cytoplasm in the massive wall thickening, and the relationship between the plasma membrane (arrow) and the membrane (arrowhead) which encloses the electron-lucent material in the cytoplasm.  $\times 27,000$

Fig. 11. Aleurone cells at the crease of a 15-day-caryopsis of greenhouse-grown barley cv. Midas. Polysomes are plentiful in the cytoplasm. Inside the plasma membrane there are microtubules (arrow) and there is electron-dense material (arrowhead) between the plasma membrane and the electron-lucent wall thickening.  $\times 17,000$



Figs. 10 and 11

dense cytoplasm but appear to be in definite configurations and not randomly distributed as in the crease aleurone cells of younger caryopses. The cytoplasm also contains vesicles which have the same electron opacity as the wall material present just outside the plasma membrane.

By 25 days after anthesis the lumina of the crease aleurone cells are surrounded by walls composed of irregular lamellae and separated by large regions of homogeneous and moderately electron-dense material. Small electron-opaque globules line the boundary between the cell walls and the



Fig. 12. Aleurone cells bordering the endosperm cavity (*ec*) at the crease of a 25-day-caryopsis of greenhouse-grown barley cv. Julia. The cells are separated by homogeneous material (*im*).  $\times 3,000$

intercellular material (Fig. 12). In 35-day-caryopses the crease aleurone cells are widely separated, the intercellular material staining lightly in toluidine blue (Fig. 7).

From about 20 days after anthesis the apparent division of the contents of the crease aleurone cells into two phases becomes less marked. However, the endoplasmic reticulum in these cells is extensive and is divided into many distended cisternae. Some of the mitochondria have cup- and ring-shaped profiles (Fig. 12) and are similar to the mitochondria found in the meristem of *Anthoceros laevis* and interpreted by MANTON (1961) as being stages in the formation of new mitochondria. Some plastids contain starch but vacuoles containing electron dense inclusions similar to those described by MORRISON *et al.* (1978), were observed only in the 35-day-barley caryopses.

Between the layer of aleurone cells lining the endosperm cavity and the large cells of the starchy endosperm there is a layer of cells with transverse wall thickenings which stain darkly with toluidine blue (Fig. 7).

### 3.3. Aniline Blue Induced Fluorescence.

The observations described will be those obtained with dye solution (4). The fluorescence observed with dye (2) was similar to that with dye (4), though less intense, while the fluorescence observed with dyes (1) and (3) was virtually the same as that in the control sections mounted in buffer.

At all stages of development from 15 to 35 days after anthesis the nucellar projection exhibited pale greenish-yellow fluorescence when mounted in buffer only, and some regions of yellow fluorescence when mounted in aniline blue. In the youngest caryopses examined, the aniline blue induced fluorescence was confined to the band of cells bordering the endosperm cavity, but in 20-day-caryopses the cell walls which fluoresced with aniline blue were on the outer and inner edges of the lobes of the nucellar projection. In 25- to 35-day-caryopses aniline blue induced fluorescence appeared in a band midway between the chalazal zone and the endosperm cavity, the intensity increasing at the sides and decreasing in the core as the age of the caryopsis increased. In 15-day-caryopses the crease aleurone showed no fluorescence when mounted in buffer but the thick walls of these cells exhibited intense aniline blue induced fluorescence. This aniline blue induced fluorescence in the walls of crease aleurone cells was more diffuse and much less intense in 20- to 25-day-caryopses and could not be detected in 35-day-caryopses.

### 3.4. Alcian Blue Staining

Blue-green staining was first observed in 15- to 20-day-caryopses between the two newly-separated lobes of the nucellar projection. In slightly older caryopses, material at the edge of both the nucellar projection and the crease aleurone stained blue-green. In 35-day-caryopses a band of cells on the periphery of the nucellar projection stained blue-green together with cells in the lateral part of the crease aleurone and the material occupying the endosperm cavity. In 40-day-caryopses the nucellar projection is short and all of it gave a very marked blue-green colour with alcian blue.

## 4. Discussion

The cells of the nucellar projection undergo a sequence of changes during the five weeks after anthesis. In 10- to 15-day-caryopses, cells having features characteristic of transfer cells (GUNNING and PATE 1974) are found close to the endosperm cavity. Beside these transfer cells, and often apparently between them and the endosperm cavity are cells with massively thickened walls. These cells may previously have possessed only wall protuberances. As the cells bordering the endosperm cavity degenerate the cells outside them elongate and acquire the characteristics of transfer cells. These in turn accumulate additional wall thickening and only the cells in the core of the nucellar projection retain transfer cell characteristics to the end of the grain-filling



period. Transfer cells occur in situations where solutes move from the symplast to the apoplast (GUNNING 1977). It is therefore possible that in the developing barley caryopsis, solutes from the phloem move symplastically through the chalaza and then into the apoplast via transfer cells in the core of the nucellar projection.

The cells of the nucellar projection bordering the endosperm cavity break down, and thus presumably provide nutrients for the developing endosperm. In the micropylar endosperm of *Capsella* (SCHULZ and JENSEN 1974) and the haustorial cell of *Orobanch*e (DÖRR and KOLLMANN 1974) stacked endoplasmic reticulum is thought to be involved in the secretion of enzymes which break down neighbouring cells. BAL and PAYNE (1972) found similar configurations of endoplasmic reticulum associated with cell wall breakdown in quiescent root meristems of *Allium cepa*. It seems likely, therefore, that the stacked endoplasmic reticulum found in some cells on the periphery of the nucellar projection is responsible for the secretion of enzymes which break down adjacent nucellar cells and, as in the cases cited above, release materials which are subsequently metabolized by neighbouring tissues.

Staining with alcian blue indicates that mucopolysaccharides are present in the endosperm cavity. It has been suggested that in mammals mucopolysaccharides control the level of electrolytes and water in extracellular fluids and impede the diffusion of larger molecules (BRIMACOMBE and STACEY 1964, SPIRO 1969). If the mucopolysaccharides present in the crease region of barley caryopses have similar properties they could play an important part in determining the chemical composition and osmolarity of the environment in which the endosperm develops.

In 10- to 15-day-caryopses the material which lies inside the massive cell walls of the crease aleurone cells appears to be composed of islands of cytoplasm bounded by membranes. In the salt glands of *Spartina foliosa*, LEVERING and THOMPSON (1971) found that in cells which had wall protuberances, the membranes surrounding the protuberances extended into the cytoplasm and partitioned it, thus giving rise to quite large extra-cytoplasmic volumes. In *Capsella*, continuity of the plasma membrane and endoplasmic reticulum has been observed along wall projections in the micropylar endosperm (SCHULZ and JENSEN 1974). If the membrane surrounding the islands of cytoplasm is plasma membrane, or has continuity with the plasma membrane, then the crease aleurone cells of young barley caryopses offer a very large surface area of membrane across which solutes could move when transferring from apoplast to symplast. Alternatively, the plasma membrane may be independent of the endoplasmic reticulum and its amplification only that due to the irregular outline of the thickened wall. The inflated and interconnected cisternae of endoplasmic reticulum may in that case function primarily in the synthesis and/or storage of carbohydrate destined for wall material. Obviously, a more detailed study is required in order to elucidate the inter-relationships and functions



of the various parts of the endomembrane system of these crease aleurone cells.

The considerable variation between the four samples of aniline blue dye confirms the findings of SMITH and McCULLY (1978) that the fluorochrome responsible for the callose reaction is a highly variable component of dye batches of water soluble aniline blue. In this context it is worth noting that W. S. Simpson & Co. have supplied aniline blue dyes to Polysciences Inc., BDH Ltd. and Raymond Lamb (W. S. Simpson & Co., personal communication) and so the differences observed by SMITH and McCULLY between dyes from Polysciences Inc., and BDH Ltd. may be characteristic only of the particular batches used.

The massive wall-thickening of the crease aleurone cells in young caryopses, and of certain cells of the nucellar projection which lie between transfer cells and the endosperm cavity at all stages of development, is electron-lucent, and these tissues give intense bright yellow fluorescence with aniline blue. Cell wall material with these characteristics is generally referred to as "callose" even though it cannot always be identified as  $\beta$ -1,3-glucan (SMITH and McCULLY 1978). Callose formed in response to wounding and during the growth of pollen tubes appears to have a plugging effect (HESLOP-HARRISON 1966). In studies on developing pollen it has been shown that glucose and acetate enter the meiocyte and young microspores while they are invested in an intact callose wall, but ( $2\text{-}^{14}\text{C}$ ) thymidine, fluorescein diacetate and  $^3\text{H}$ -phenylalanine do not (HESLOP-HARRISON and MACKENZIE 1967, KNOX and HESLOP-HARRISON 1970, and SOUTHWORTH 1971). HESLOP-HARRISON (1964) had earlier proposed that the callose wall preserved the autonomy of the haploid nucleus by preventing the entry of certain molecules from the parent plant. If the callose in the crease region of developing barley caryopses has a similar effect on permeability, and if it is assumed that all materials destined for the endosperm enter through the crease (FRAZIER and APPALANAIDU 1965), then, the developing endosperm will be isolated from substances of parental origin such as hormones, which might influence its development. In addition, as MORRISON and O'BRIEN (1978) have suggested for very young wheat caryopses, callose may protect the developing endosperm from enzymes which break down the nucellar cells.

However, callose is not always associated with situations where permeability is considerably reduced and SMITH and McCULLY (1978) point out that the two properties by which it is identified, *i.e.*, electron-lucency and aniline blue fluorescence, are characteristics of an open wall construction which allows penetration of resin and dye respectively. The callose in the cell walls of the crease aleurone and the nucellar projection could thus be interpreted as providing broad apoplastic pathways or temporary storage sites for materials which leave the symplast of the parent plant in the transfer cells of the nucellar projection.

Another characteristic of callose is its rapid synthesis and breakdown (CURRIER 1957). Its significance in the crease region may therefore lie in the early stabilization of the morphology of the endosperm in this transport zone (EVERS 1970) and the ease with which it can be used at a later stage of development to provide a source of carbon and energy (DWYER and SMILLIE 1971). Clearly, much more work is needed before the role of callose in the crease region of developing cereal caryopses can be established.

### Acknowledgements

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## THE INFLUENCE OF NUTRITION ON EMBRYO DEVELOPMENT AND GERMINATION

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### SUMMARY

The development of immature isolated barley embryos in response to variation in the nutrient supply is described. Using *in vitro* culture techniques it has been found that the nature of the nitrogen source, the sucrose concentration and other factors influence the survival, development and onset of germination of immature cultured embryos.

### INTRODUCTION

The *in vitro* culture of excised embryos on defined media is a technique of value in the production of seedlings from hybrid crosses where *in vivo* development is prevented by, for example, incompatibility between embryo and endosperm during grain development. It is also an attractive technique to use in the study of the control of embryo growth, development and germination. Recent studies (Cameron-Mills and Duffus 1977) have shown that 'normal' development and germination of barley embryos, as small as 0.25 mm in length, can be achieved in culture. Normality was assessed by reference to a series of morphological markers relating to the embryo under *in vivo* conditions. A range of culture media was used but only one - originally described by Norstog (1973) (Medium II) - supported 'normal' development and germination of the smallest embryos. In the present work, an early culture medium devised by Norstog and Smith (1963) (Medium I), on which only large, mature (1.2 mm) barley embryos developed normally, has been used. By comparing this medium with Medium II and by observing the effect of various additions to it, a number of possible factors involved in embryo development and germination have been identified.

### MATERIALS AND METHODS

The two-row barley *Hordeum distichum* L. var Julia was grown under greenhouse conditions with day length extended to 18 h using mercury vapour lamps. Embryos were excised under sterile conditions and grown in culture as previously described (Cameron-Mills and Duffus, 1977). They were transferred to test tubes containing 5 ml media at the onset of germination and subsequently grown in an illuminated growth cabinet with an 18 h day. Ammonium ion was assayed in endosperm extracts using glutamate dehydrogenase (Tabor, 1970). Free amino acids were assayed using ninhydrin (Chrispeels and Boulter, 1975) after extraction of endosperms in 7.5 per cent sulphosalicylic acid. Endosperm sucrose was assayed enzymatically using glucose oxidase after conversion to glucose and fructose using invertase (Johnson *et al*, 1964).

## RESULTS AND DISCUSSION

A major problem faced in the *in vitro* cultivation of immature barley embryos is the phenomenon of precocious germination. Thus, immature embryos isolated and placed on agarose growth media may germinate immediately. The inability of such embryos to germinate normally and grow into healthy plants is attributed to the premature cessation of embryogenesis and failure to complete the attendant development of shoot and root primordia.

In the mature grain at the onset of germination, the scutellum of the embryo measures about 3.5 mm by 2.5 mm (length x width: 8.75). *In vitro* culture media based on Norstog's Medium I have been tested for their ability to delay germination and promote embryogenesis. The characteristics of their subsequent germinative growth, namely the rate of leaf and root initiation and growth, as compared with that of germinating grains, have been used as an indicator of normal embryogenesis.

Norstog's Medium I will not support the growth and germination of embryos isolated less than 18 days after anthesis (Fig. 1). Eighteen day embryos will grow and may attain a mature size within 8 days of culture. However, several abnormalities can be discerned in their germinative growth (Fig. 4) when compared with that of the native embryo, including an early cessation of coleoptile elongation, delayed leaf growth and restricted root initiation and growth. One may conclude that normal embryogenesis did not occur during this period of pre-germinative growth.

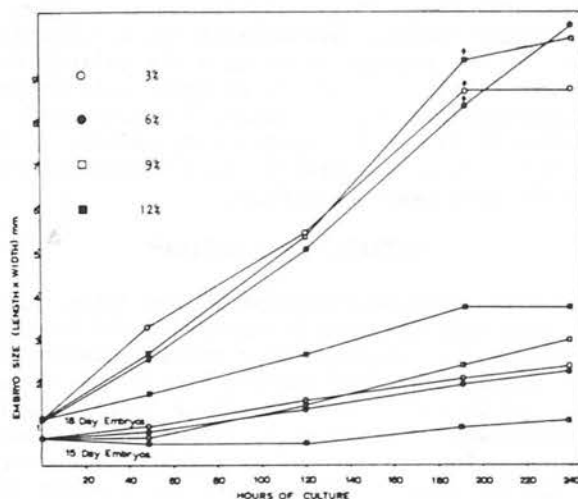


FIG. 1. Effect of sucrose (3-12%) on embryo growth in culture.



It has previously been observed that high sucrose concentrations may delay the onset of germination (Cameron-Mills and Duffus, 1977). It has also been found that increased sucrose concentrations from 3% to 9% cause a progressive decline in the germination growth rate, while embryos grown in 12% sucrose completely fail to germinate (results not shown). Figure 1 shows that in 18 day old embryos, 12% sucrose delays the onset of germination and prevents pre-germinative growth. Younger embryos failed to grow satisfactorily at all sucrose concentrations.

From Figure 2 it is clear that coconut milk supplemented media, in contrast to sucrose-enriched media, enhance precocious germination. Only older 25 day embryos grown at the higher coconut milk concentrations of 10-15%, reached a mature size prior to germination, their development being considerably faster than on Norstog's Medium I. Despite their limited embryogenesis, germination of 20 day embryos resembled more closely that of the native embryo (Fig. 4), the balance of shoot and root growth being largely restored. It is difficult, however, to identify the factors responsible for the improved growth on coconut milk without a complete analysis of its components.

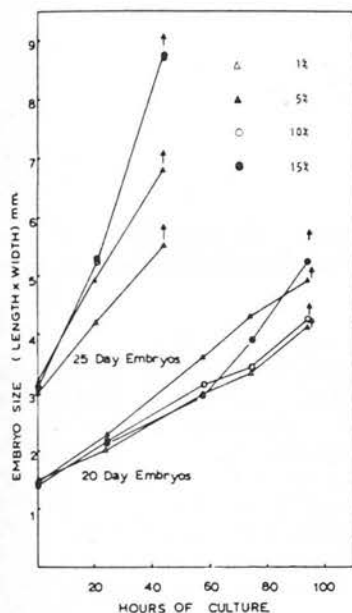


FIG. 2. Effect of coconut milk (1-15%) on embryo growth in culture.

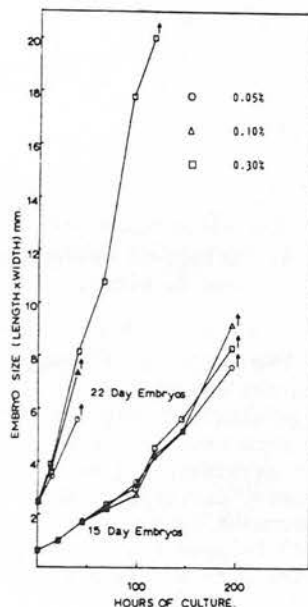


FIG. 3. Effect of casein hydrolysate (0.05-0.3%) on embryo growth in culture.

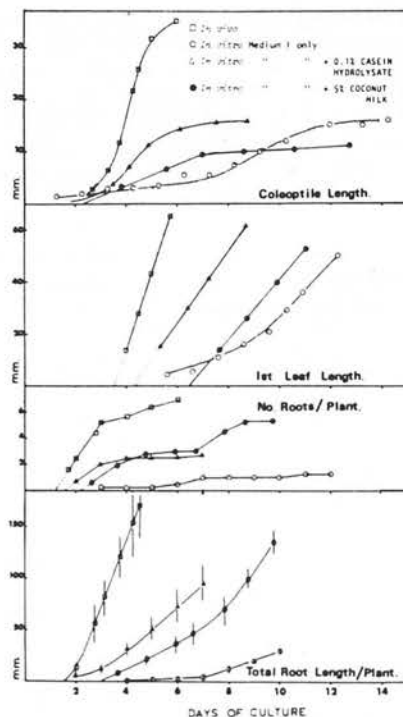


FIG. 4. Effect of various additions on barley embryo germination *in vivo* and *in vitro*.

The addition of casein hydrolysate to Norstog's Medium I, in place of amino and organic acids, had the most marked effect on both embryogenesis and germination (Fig. 3). Casein hydrolysate at 0.1% stimulated a rapid growth to maturity in embryos as young as 15 days and further enhanced their germinative growth, even more than coconut milk (Fig. 3,4). Rates of embryo development were comparable to those on Norstog's Medium II (Cameron-Mills and Duffus, 1977). Higher casein hydrolysate levels (0.3%) delayed the onset of germination of 22 day embryos and growth continued to a size far exceeding that of the mature normal embryo.

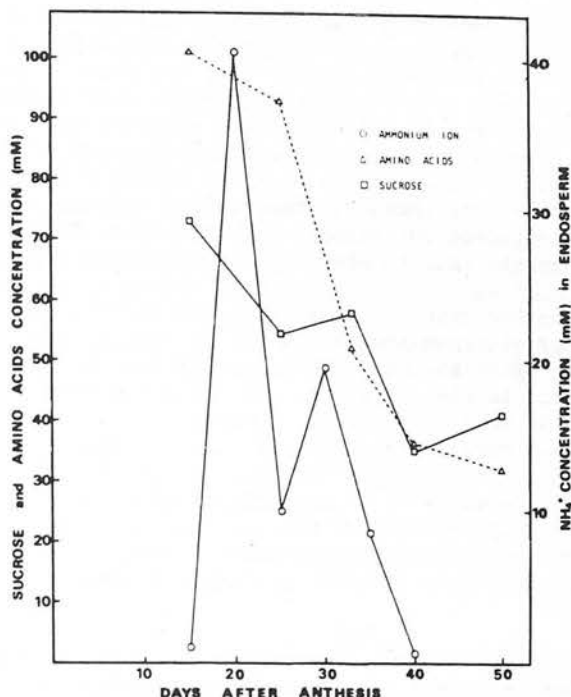


FIG. 5. Variation in concentration of ammonium ion, amino acids and sucrose during endosperm development in barley.

It is clear from these embryo culture experiments that the development and germination of embryos is strongly influenced by their growth environment. A graph of the concentration of certain key metabolites found in the developing endosperm (Fig. 5) reveals the considerable changes in the concentration of nutrients available to the embryo during embryogenesis. Both free amino acid and sucrose concentrations fell throughout endosperm development from about 100mM to 32mM and 73mM (2.4%) to 41mM (1.4%) respectively, during the period of 15 to 50 days after anthesis. In contrast, ammonium ion increased rapidly from very low levels at 15 days to a maximum value of 40mM at 20 days, falling off thereafter to zero by 35 days after anthesis. Such variation must place restrictions on the initiation and duration of embryo development. The absence of such changes in growth environment of the cultured embryo may

remove the restrictions on growth and germination observed *in vivo*, and thereby allow the premature progress of the embryo from one growth phase to the next. While low water availability may prevent germination in the ripened grain, a high sucrose (348mM) or amino acid (20mM casein hydrolysate) concentration will delay *in vitro* germination. Such non-specific concentration effects may primarily reflect the role of osmotic potential in the control of germination, as has been observed in cultures of isolated cotton embryos (Mauney, 1961). High sucrose levels are known to inhibit cell elongation but promote cell division (Raghavan, 1976), which may well explain the failure of cultured embryos to grow on 12% sucrose.

The most marked differences between the *in vivo* and *in vitro* nutrients lie in the major nitrogen source (Table 1). At the stage of embryo isolation, 20 days after anthesis, both ammonia ion and free amino acids reach peak levels in the endosperm, and these considerably exceed their concentrations in Norstog's media. A study of the nitrogen metabolism of developing barley grains has indicated that inorganic nitrogen is supplied to the grain as ammonium rather than nitrate (Duffus and Rosie, 1978). Thus the poor performance of 15 day embryos on Norstog's Medium I may result from the preponderance of nitrate over ammonium ions. This view is supported by the dramatic improvement of embryo survival and growth on Norstog's Medium II where ammonium ion replaces nitrate as major nitrogen source.

Table 1. Some differences in composition of Embryo Culture Media and Barley Endosperm (20 days after anthesis).

Component (mM)	Endosperm	Norstog's Media		Casein hydrolysate (0.1%)
		I	II	
Sucrose	64	87	87	-
Ammonia	40	1.2	12	-
Nitrate	0	4	0	-
Total Amino Acids	97	7.6	3.69	6.54

The rationale behind the formulation of the amino acids in Norstog's media is unclear since it bears little relationship to that of the developing endosperm (Brandt, 1976). The presence of additional amino acids in casein hydrolysate may partly explain its ability to enhance embryogenesis.

#### CONCLUDING REMARKS

Very young embryos can grow and develop into normal plants on defined and fairly simple media. We therefore consider that pre-harvest sprouting may be controlled by variation in concentration of some simple metabolite or metabolites, and may merely be a function of endosperm osmotic potential. The techniques of embryo culture described here have not so far been extended to include a period of dehydration and dormancy, although there is no experimental reason why this should not be possible. At present the embryos do not become dormant, but germinate immediately they reach a critical size on a medium of low sucrose concentration. By following normal embryo growth

on Medium II by a period of gradual dehydration, this technique might be used to study the factors regulating dormancy and germination.

#### ACKNOWLEDGEMENT

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## Endosperm cell number in barley

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Grain weight is one of the major components of grain yield and it has been suggested that in wheat, final grain weight may be a function of endosperm cell number<sup>1</sup>. If grain weight is to be increased by increasing the rate and/or duration of endosperm cell division, it is important to establish the stage of development at which maximum endosperm cell number is attained. Evers<sup>2</sup> reported that no cell division occurred in the endosperm of wheat later than 16 days after anthesis and other workers<sup>3,4</sup> have come to a similar conclusion using cell counts. However, biochemical analyses<sup>5</sup> have shown that the maximum DNA content of wheat endosperm is not reached until 25 days after anthesis and both Donovan<sup>6</sup> and Radley<sup>7</sup> have suggested that endosperm cell division may continue until about 30 days after anthesis. We have estimated the number of endosperm nuclei in developing caryopses of barley and have examined transverse sections of endosperms of different ages. We report here that cell division in barley endosperm continues until 28–30 days after anthesis.

In the present investigation, as in previous work in this laboratory<sup>8</sup>, barley (*Hordeum vulgare* var. *distichum* L. cv. Midas) was grown in a greenhouse, and caryopsis ages were estimated on a scale based on the normal development of field-grown barley in Scotland, which takes 60 days from anthesis to harvest-ripeness. The parameters used to estimate developmental age include pericarp shape and colour, endosperm shape and colour, and embryo length. The middle three or four grains were removed from each of several of the first-maturing ears in two or more pots. The developmental age of each caryopsis was determined and the endosperm dissected out, taking care to remove the testa and embryo and leave the aleurone intact. It was not possible to sample caryopses older than 33 'days' after anthesis because at this stage of development, the aleurone adheres to the testa and peels off with it. Endosperms were fixed overnight in 1:3 v/v acetic acid/ethanol and then stored in 70% ethanol. Endosperms of the same developmental age were pooled and three samples of 10 were

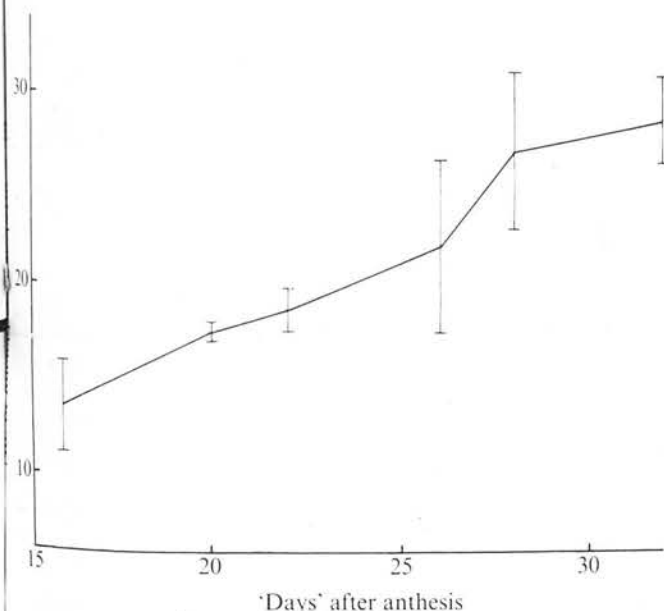


Fig. 1 The number of nuclei per endosperm during barley caryopsis development. Bars indicate confidence limits at  $P = 0.05$ .

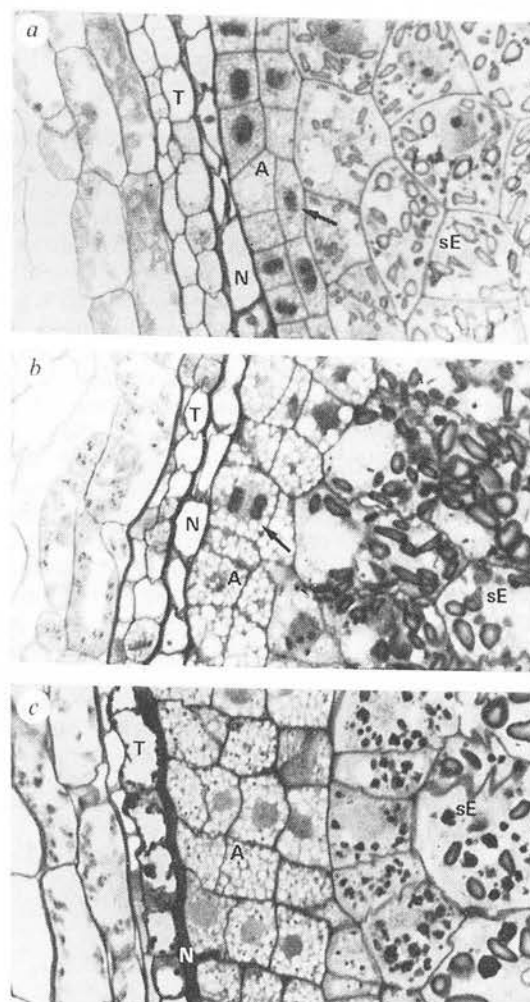


Fig. 2 Transverse sections of three barley caryopses cut at mid-grain. The material was fixed in glutaraldehyde, post-fixed in osmium tetroxide, embedded in Epon and 1.5- $\mu$ m sections were stained in toluidine blue. The tissues shown above are from the lateral part of the caryopsis. Cells undergoing division (arrows) can be seen in the aleurone layer (A) of 15-'day' and 23-'day' caryopses. These sections illustrate the state of development of the nucellus (N), testa (T) and starchy endosperm (sE) as the number of endosperm nuclei increases (Fig. 1). a, 15-'day'; b, 23-'day'; c, 33-'day'.  $\times 400$ .

stained using Feulgen reagent and digested in cellulase<sup>9</sup>. Cells were suspended in 0.5 M sorbitol and nuclei were counted in a haemocytometer. Caryopses from the same ears were prepared for light microscopy<sup>8</sup>.

The number of nuclei per endosperm increased during the period 16–28 'days' after anthesis (Fig. 1). Transverse sections of mid-grains (Fig. 2) show that the aleurone layer of a 15-'day' caryopsis is one to two cells thick, with cell division occurring in both anticlinal and periclinal planes. The cells in the inner and outer layers of the testa are similar in size and the cells of the nucellus are fully expanded and thin-walled. The endosperm of the 15-'day' caryopsis is flat and greyish-white, and the embryo cannot be seen with the naked eye. In 23-'day' caryopses the aleurone is two to three cells thick and cell division can be observed (Fig. 2). The cells in the outer layer of the testa are thinner than those in the inner layer, and the nucellar cells are thick-walled. At this stage the endosperm is white and rounded and the embryo is  $\sim 0.25$  mm long. By 33 'days' after anthesis, three to five layers of aleurone are established and clearly differentiated from the sub-aleurone and the starchy endosperm. The inner testa cells are large and have osmiophilic



contents, and the nucellus is represented only by a layer of compressed wall material. In 33-'day' caryopses the endosperm is yellowish, firm and well rounded, the embryo is ~2.1 mm long and the aleurone layer peels off with the testa.

These observations on developing barley caryopses differ from those reported for wheat in that the number of nuclei per endosperm in barley increased up to about 30 'days' after anthesis, whereas in wheat, increase in the number of nuclei per endosperm stopped 16–20 days after anthesis<sup>3,4</sup>. In addition, evidence of endosperm cell division was observed at a later stage of development in barley than that reported for wheat<sup>2</sup>. These differences may be due to: (1) some assays being done on wheat endosperms contaminated with testa or stripped of their aleurone cells; (2) comparisons being made on the basis of strict chronological age, taking no account of variation in morphological development due to environmental factors; (3) the fact that the aleurone layer in wheat caryopses is only one cell thick whereas that in barley is at least three cells thick and so cell division in barley may continue for some time after the last starchy endosperm cells have been initiated. Note, however, that there is evidence of anticlinal divisions in the meristematic aleurone of 23-'day' barley endosperms and the cells thus produced presumably contribute to an increase in the surface area of the endosperm and hence to an increase in endosperm volume. It seems unlikely that similar anticlinal divisions in the aleurone of wheat cease as early as 16 days after anthesis<sup>2</sup>.

Perhaps, therefore, more attention should be paid to the suggestions of Donovan<sup>6</sup> and Radley<sup>7</sup> that cell division in wheat endosperm continues up to 25–30 days after anthesis and to

Sandstedt<sup>10</sup>, who observed that cell divisions occurred in the endosperm of wheat, grown in Nebraska, up to 16 days after anthesis, that is, half-way between anthesis and maturity in those climatic conditions. If attempts are to be made to increase the weight of cereal grains by applying chemicals which increase endosperm cell number, it is important to establish the pattern of cell division in the developing grain. The timing of the application of any such chemicals could be particularly critical in barley because it seems that cell division taking place after a certain stage of development produces mainly aleurone cells and therefore does not provide further capacity for carbohydrate storage. Thus, stimulation of endosperm cell division early in development could increase the yield of carbohydrate, whereas stimulation of cell division at a later stage could improve the nutritional quality of the grain by increasing the yield of the proteins, fats, minerals and vitamins which are found in aleurone cells.

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## GROUP 2

### Control of Germination

# A Possible Role for Cyclic AMP in Gibberellic Acid Triggered Release of $\alpha$ -Amylase in Barley Endosperm Slices

$\alpha$ -Amylase is released by the aleurone layer into the starchy endosperm during barley germination. In the de-embryonated grain this release can be effected by the addition of the plant hormone gibberellic acid and has been shown to involve a de novo synthesis of the enzyme<sup>1</sup>.

3' 5' Cyclic adenosine monophosphate (cyclic-AMP) has recently been implicated as an intermediate in the action of a number of hormones<sup>2-4</sup>. In addition it has been shown to cause secretion of  $\alpha$ -amylase from the rat salivary gland<sup>5</sup>.

The concentration of cyclic-AMP in the cell is a function of its rate of formation from ATP by adenylyl cyclase and its rate of breakdown via a specific 3' 5' cyclic diesterase to AMP. Aminophyllin (theophyllin) is a competitive inhibitor<sup>6</sup> of the diesterase and has been shown to cause an intracellular accumulation of cyclic-AMP<sup>7</sup>. Thus any effect due to the action of cyclic-AMP might be expected to be increased in the presence of this inhibitor.

The present work indicates that cyclic-AMP can trigger  $\alpha$ -amylase release in barley endosperm slices. It has further been shown that aminophyllin has a similar effect.

The barley used was a sample of Maris Baldric, dehulled by treatment with 50%  $H_2SO_4$  and stored at 4°C. 2 mm endosperm slices in groups of 10, were incubated for 18 h at 25°C in 4 ml of solution as indicated below. The slices were preincubated for 1½ h at 25°C with 4 ml distilled water which was decanted off just before addition of the solutions. 1 ml M NaCl was added to the solutions before homogenizing in a glass (hand) homogenizer. The homogenates were left to stand for 1 h at room temperature before centrifuging. (M.S.E. bench centrifuge speed 10.)  $\alpha$ -Amylase activity in the supernatant was assayed at 25°C by the iodine-dextrin colour method of BRIGGS<sup>8</sup>.

The results, tabulated below, indicate the relative activities of  $\alpha$ -amylase released, expressed in enzyme units/g fresh weight of tissue as described by DUFFUS.<sup>9</sup>

Cyclic AMP at a concentration of  $10^{-5}M$  stimulates gibberellic acid controlled release of  $\alpha$ -amylase. Aminophyllin, on the other hand, has no significant effect. This suggests that the local concentration of cyclic AMP is so high that effective competition with aminophyllin is prevented.

Enzyme release due to the action of cyclic AMP and aminophyllin, separately and in combination, is about 10% that with gibberellic acid. The fact that there is no additive action is noteworthy. Furthermore,  $\alpha$ -amylase release due to cyclic AMP increases with increase in concentration reaching a value of 111 enzyme units/g fresh weight of tissue at  $10^{-3}M$  cyclic AMP. No  $\alpha$ -amylase release was observed using adenosine 5' monophosphate (5'-AMP) at a concentration of  $10^{-5}M$ .

The results would suggest that cyclic AMP is a possible intermediate between gibberellic acid stimulus and  $\alpha$ -amylase release. Aminophyllin acts presumably through inhibition of the 3' 5' cyclic diesterase thus preventing cyclic AMP breakdown. (Neither cyclic AMP nor aminophyllin has any direct effect on the activity of  $\alpha$ -amylase in the assay system used.)

Part of the action of gibberellic acid may be analogous to that of other hormones which apparently act by stimulation of a membrane bound adenylyl cyclase<sup>10,11</sup> to give an increase in intracellular cyclic AMP concentration. That gibberellic acid may have some effect on the membrane was suggested by MACLEOD et al.<sup>12</sup>

It is probable, however, that gibberellic acid does more than increase the cyclic AMP level since the maximum

rate of enzyme release with cyclic AMP is only 25% of that with gibberellic acid. This of course may be merely a function of the relative ease of penetration of the 2 compounds.

How this cyclic AMP effect relates to the stimulation of nucleic acid and protein synthesis by gibberellic acid<sup>1,13</sup> remains to be elucidated<sup>14</sup>.

Relative activities of  $\alpha$ -amylase released by action of gibberellic acid, cyclic AMP and aminophyllin

Addition	$\alpha$ -amylase activity in E U/g fresh weight
Gibberellic acid ( $10^{-5}M$ )	421 $\pm$ 80
+ cyclic AMP ( $10^{-5}M$ )	711 $\pm$ 50
+ Aminophyllin ( $10^{-3}M$ )	385 $\pm$ 170
Cyclic AMP ( $10^{-5}M$ )	36 $\pm$ 18
+ Aminophyllin ( $10^{-3}M$ )	24 $\pm$ 4
Aminophyllin ( $10^{-3}M$ )	19 $\pm$ 7
Cyclic AMP ( $10^{-3}M$ )	111 $\pm$ 25
5'-AMP ( $10^{-5}M$ )	< 5
Distilled water	< 5

Each result is the mean of at least 3 experiments  $\pm$  standard deviation

**Résumé.** L'adénosine monophosphate cyclique peut agir comme intermédiaire dans la synthèse, contrôlée par l'acide gibberellique, de l' $\alpha$ -amylase ( $\alpha$ -1,4-glucan-4-glucanohydrolase) dans des tranches de grains d'orge sans embryon.

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## Inhibition of Gibberellic Acid Biosynthesis by Chorionic Gonadotrophin During Cereal Grain Germination

It has been suggested<sup>1,2</sup> that a gonadotrophin-like growth regulating factor may be present in plants. It was proposed that this factor might be involved in the regulation of endogenous gibberellin levels since these were reportedly lowered in the presence of human chorionic gonadotrophin (HCG). However, they were assayed by treating embryoless barley endosperm halves with extracts and measuring reducing sugar release<sup>3</sup>. Intrinsically this method is not entirely satisfactory<sup>4,5</sup> and, since it was found at the same time that HCG would inhibit reducing sugar release in response to gibberellic acid (GA), the exact significance of the results must be open to doubt. On the other hand, since no polypeptide or glycoprotein hormone-like substance has been reported in plants, it seemed worthwhile to clarify the situation. For this purpose the germinating barley grain was chosen since in it gibberellin synthesis and secretion (in the embryo) are readily separated from gibberellin action (on the aleurone).

The barley used was a sample of Proctor, dehusked by treatment with 50%  $H_2SO_4$  and stored at 4°C. Whole grains or 2 mm endosperm slices were weighed in groups of 10 and incubated for 26 h at 25°C in 4 ml of solution as indicated below. 1 ml *M* NaCl was added to the solutions before homogenizing in a glass (hand) homogenizer. The homogenates were left to stand for 1 h at room temperature before centrifuging (MSE bench centrifuge, 5 min speed 10).  $\alpha$ -Amylase activity in the supernatant was assayed at 25°C by the iodine-dextrin colour method of BRIGGS<sup>6</sup>.

The results, tabulated below, indicate the relative activities of  $\alpha$ -amylase released in terms of the rate constants for the reaction and expressed in arbitrary units (AU) per g fresh weight as described by DUFFUS<sup>7</sup>.

Human chorionic gonadotrophin, Stock No. CG-B from the Sigma Chemical Corporation at a specific activity of

2800 IU/mg was used. This preparation contained no additives but its low specific activity indicates that it may contain albumin and denatured HCG as impurities.

The results show that HCG at a concentration of 7 IU/dish will inhibit  $\alpha$ -amylase production in intact germinating grains by 50%. Inhibition is virtually complete at a concentration of 140 IU/dish. Gibberellic acid dependent  $\alpha$ -amylase synthesis in 2 mm endosperm slices is not inhibited by levels of HCG up to 280 IU/dish.  $\alpha$ -Amylase activity is unaffected by HCG.

Since HCG has no effect on the stimulated rate of  $\alpha$ -amylase synthesis in intact grains incubated with  $GA_3$ , where the  $GA_3$  must be transported from the micropile to the aleurone, it may be concluded that transport of  $GA_3$  from the embryo to the aleurone layer is not inhibited. HCG must therefore inhibit either the synthesis or secretion of  $GA_3$ .

This is not the first report of an animal hormone eliciting a biochemical response in plants since ecdysone, the insect growth hormone stimulates growth in dwarf pea seedlings in a manner similar to that of  $GA_3$ <sup>8</sup>. There may well be others and it is possible that bioassays much cheaper and simpler than those currently in use for animal hormones might be devised using plant tissues. A correlation between the various activities of the hormone would have to be demonstrated in this event.

So far no specific factor controlling GA biosynthesis in plants has been described. Environmental factors such as light and cold influence GA biosynthesis but not with any degree of specificity. BRIAN<sup>9</sup> has postulated that GA biosynthesis is controlled in vivo by the phytochrome system. It is unlikely however, that phytochrome is uniquely concerned in GA biosynthesis as it has been implicated in a wide range of physiological phenomena.

If, as we suggest, a regulatory substance similar to HCG and controlling GA biosynthesis, is present in germinating barley then it may form part of a general mechanism governing GA levels in plants. For example, dwarfing of plants, frequently a result of a lowered GA production, may be caused by an overproduction of this HCG-like regulatory substance. Other basic plant processes in which GA is known to be concerned, such as abscission<sup>10</sup>, senescence<sup>11</sup> and dormancy<sup>12</sup> may also involve this substance.

Relative activities of  $\alpha$ -amylase released in terms of the rate constants for the reaction in intact barley grains and endosperm slices

Material	Addition	$\alpha$ -amylase activity in AU/g fresh wt. <sup>a</sup>
Intact grains	Distilled water	0.128 $\pm$ 0.02
	+ 140 IU <sup>b</sup> HCG	< 0.02
	+ 70 IU <sup>b</sup> HCG	0.05 $\pm$ 0.02
	+ 42 IU <sup>b</sup> HCG	0.04 $\pm$ 0.02
	+ 14 IU <sup>b</sup> HCG	0.064 $\pm$ 0.03
	+ 7 IU <sup>b</sup> HCG	0.061 $\pm$ 0.035
	+ 3 IU <sup>b</sup> HCG	0.108 $\pm$ 0.03
	5 $\times$ 10 <sup>-8</sup> M $GA_3$	0.294 $\pm$ 0.08
Endosperm slices	+ 280 IU HCG	0.308 $\pm$ 0.08
	Distilled water	< 0.02
	5 $\times$ 10 <sup>-8</sup> M $GA_3$	0.126 $\pm$ 0.01
	+ 280 IU HCG	0.115 $\pm$ 0.02
	+ 140 IU HCG	0.129 $\pm$ 0.03
	5 $\times$ 10 <sup>-8</sup> M $GA_3$ + 280 IU HCG added just before assay	0.126 $\pm$ 0.02

Each result is the mean of at least 3 experiments  $\pm$  S.D. <sup>a</sup> AU, arbitrary units. <sup>b</sup> IU, international units of HCG.

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Finally, it would be interesting to know the effect of HCG on GA production by *Fusarium moniliforme*, the original source of GA<sup>13</sup>; if inhibition occurs, conceivably this may prove the ideal system for elucidating the mode of action of HCG at the molecular level<sup>14</sup>.

*Résumé.* La gonadotrophine chorionique peut arrêter la production de l' $\alpha$ -amylase pendant la germination des grains intacts d'orge. Par contre, la synthèse de l' $\alpha$ -amylase contrôlée par l'acide gibberellique dans les sections de grains d'orge sans embryon n'est pas modifiée. La

gonadotrophine chorionique doit donc arrêter la synthèse ou la sécrétion de l'acide gibberellique.

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## Glutamate Decarboxylase in Barley Aleurone and its Relationship to $\alpha$ -Amylase Development During Germination

Both the enzyme glutamate decarboxylase and its product  $\gamma$ -aminobutyric acid (GABA) have been reported to occur widely in plants<sup>1</sup>. Their physiological importance has not yet been clearly defined although tracer experiments have all shown a close relationship between GABA and Krebs cycle acids<sup>1-3</sup>. GALSKEY and LIPPINCOTT<sup>4</sup> have recently reported that the synthesis of  $\alpha$ -amylase is activated in embryoless barley seeds by a number of amino acids, including glutamic and aspartic acids. GABA did not induce  $\alpha$ -amylase activity in these experiments but we considered it would be of interest to examine the effect of GABA on  $\alpha$ -amylase which was induced by use of gibberellic acid (GA)<sup>5</sup>.

The barley used was *Hordeum vulgare* L. var. Maris Otter, dehusked with 50% sulphuric acid and stored in a desiccator at room temperature. Studies of the interaction of GABA and GA were carried out using 2 mm endosperm slices, as previously described for similar studies with cyclic AMP and GA<sup>6</sup>.  $\alpha$ -Amylase activity was assayed by the iodine-dextrin colour method of BRIGGS<sup>7</sup>. The results were expressed in arbitrary units (AU)<sup>8</sup>. The effect of GABA on  $\alpha$ -amylase activity was determined in the normal assay system. A fixed amount of enzyme was preincubated for 1 h with appropriate concentrations of GABA before determining the activity. Alternatively, GABA was added to the substrate to give a suitable concentration in the final assay mixture.

Aleurone was prepared from dehusked barley germinating at 25°C using the method previously described by DUFFUS<sup>9</sup>. Samples of 50 grains showing optimum germination rates were taken and the aleurone was prepared from 2 mm slices cut from the central portion of each grain. The combined aleurone pieces were mixed with ground glass and 0.5 ml of water in a Potter type homogenizer and extracted at 4°C. The crude homogenate was centrifuged at 1000 g and 4°C for 10 min. The supernatant was assayed for glutamate decarboxylase activity. The assay system had a total volume of 0.3 ml and contained 75 mM potassium phosphate pH 6.2, 10  $\mu$ M pyridoxal phosphate, 40 mM sodium glutamate and 0.1 ml of aleurone extract. The solution minus glutamate was incubated at 25°C for

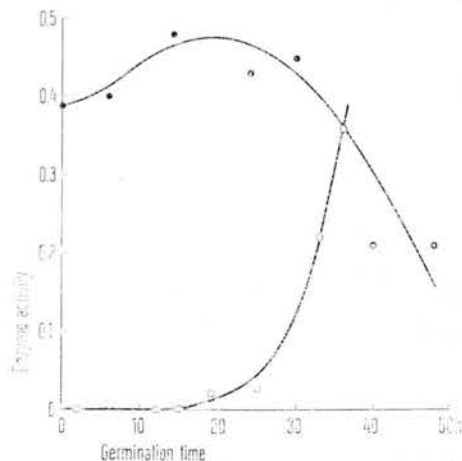
10 min when glutamate was added to start the reaction. After 30 min further incubation at 25°C, 0.03 ml of 5 N H<sub>2</sub>SO<sub>4</sub> were added to stop the reaction. The GABA formed was estimated spectrophotometrically in a neutralised aliquot of the reaction solution using enzymes prepared from *Pseudomonas fluorescens*<sup>10</sup>. In the case of each extract, the GABA present in an assay to which no glutamate had been added was measured. The difference between the two GABA contents was then taken as a measure of glutamate decarboxylase activity.

Initial experiments on the effect of GABA on  $\alpha$ -amylase activity were carried out using the system designed to measure the production of  $\alpha$ -amylase by barley aleurone under the influence of GA. The results appeared to indicate that concentrations of GABA of between 10<sup>-6</sup>M and 10<sup>-3</sup>M cause an approximately 30% decrease in  $\alpha$ -amylase production. However, further experiments showed that GABA acts directly as an inhibitor of  $\alpha$ -amylase in vitro rather than exerting an effect on the biosynthetic system. Whereas 2  $\times$  10<sup>-6</sup>M GABA was without effect on  $\alpha$ -amylase activity, a maximum inhibition of 25% was obtained at 5  $\times$  10<sup>-6</sup>M GABA with no further increase at concentrations up to 10<sup>-3</sup>M. Inhibition by GABA was found only when preincubation of GABA and enzyme was carried out; addition of a mixture of GABA and substrate to the enzyme did not result in any detectable inhibition of  $\alpha$ -amylase activity.

The glutamate decarboxylase and  $\alpha$ -amylase activities in the aleurone of optimally germinating barley samples were followed for the first 48 h of germination (Figure). Glutamate decarboxylase showed an initial slight rise in activity followed by a steep decline. The onset of this decline appears to coincide with the appearance of  $\alpha$ -amylase activity in the tissue.

Attempts were made to demonstrate the presence of GABA in barley aleurone using the specific dansylation and chromatographic method of SEILER and WIECHMANN<sup>11</sup> but the compound could not be detected at a sensitivity level of approximately 0.5  $\mu$ moles/g aleurone. If GABA is present at a level found to be effective against  $\alpha$ -amylase in vitro, e.g. 5  $\times$  10<sup>-6</sup>M, calculation suggests that the aleurone from more than a kilogram of barley may be necessary for the extraction of sufficient GABA to enable identification with present techniques.

INATOMI and SLAUGHTER<sup>1</sup> reported that they could not detect glutamate decarboxylase activity in extracts of barley endosperm but in their case whole endosperm was used and it seems probable that significant disruption and extraction of the aleurone cells was not achieved by the



Changes in activity of glutamate decarboxylase and  $\alpha$ -amylase during germination at 25°C.  $\alpha$ -amylase activity is in AU/grain and glutamate decarboxylase activity is expressed as  $\mu$ moles GABA produced/h/aleurone slice.

glutamate decarboxylase ●  
 $\alpha$ -amylase ○

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technique used. In the present paper it has been shown that water extracts of separated aleurone layers contain glutamate decarboxylase activity in easily detectable amounts. Thus barley aleurone appears to have the capacity to produce GABA as does the embryo<sup>1</sup>. It does not follow, however, that the functions of GABA are the same in both tissues: in embryos the enzyme glutamate decarboxylase increases in activity as germination proceeds and considerable quantities of GABA are synthesised<sup>1</sup>, whereas in aleurone cells the glutamate decarboxylase activity declines steeply after a few hours of germination, and GABA cannot at present be detected. It has been suggested that in the developing embryo glutamate decarboxylase and GABA are important in the build-up and functioning of the Krebs cycle<sup>1,3</sup> whereas in the aleurone cells it seems possible that GABA may serve a regulatory function.

The discovery of a possible control function for GABA in plants suggests that GABA may have the same two broad functions in plants as in animals<sup>12-14</sup>. Namely as an intermediary metabolite concerned with the functioning of the Krebs cycle and as part of a system controlling the development and functioning of the overall organism<sup>15</sup>.

**Résumé.** L'enzyme glutamate décarboxylase se trouve dans les extraits d'eau de l'aleurone de l'orge. Au début de la germination, l'activité de cette enzyme augmente,

mais après, quand apparaît l'enzyme  $\alpha$ -amylase, elle diminue. Quoiqu'au dessous d'une concentration de  $2 \times 10^{-6} M$  l'acide  $\gamma$ -amino butyrique (GABA) n'affecte pas l'activité de l' $\alpha$ -amylase in vitro, la GABA à  $5 \times 10^{-6} M$  peut provoquer une inhibition de 25% qui n'est pas modifiée par des concentrations de GABA comprises entre  $5 \times 10^{-6} M$  et  $10^{-3} M$ .

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### $\alpha$ -Amylase and its Release by Prostaglandin $F_{2\alpha}$ in Barley Endosperm Slices

3',5' cyclic AMP (cyclic AMP) has been implicated<sup>1,2</sup> as an intermediate in the action of a number of mammalian hormones. It may also have a role in gibberellic acid triggered release of  $\alpha$ -amylase during cereal grain germination<sup>3,4</sup>. It now appears that prostaglandins may be concerned in regulation of cyclic AMP levels in a variety of mammalian tissues. Most of the work reported has concentrated on the action of prostaglandins  $E_1$  and  $E_2$ . In general they decrease cyclic AMP concentration in adipose tissue and increase it in most other tissues studied<sup>5</sup>. Prostaglandin  $F_{2\alpha}$  has been reported to have no effect on adenylyl cyclase in thymic lymphocytes<sup>6</sup> or intact uterus strips<sup>7</sup>.

The present work indicates that prostaglandin  $F_{2\alpha}$  can trigger release of  $\alpha$ -amylase in barley endosperm slices. The barley used was *Hordeum vulgare* L. var. Maris Otter, dehusked by treatment with 50%  $H_2SO_4$  and stored at room temperature. 2 mm endosperm slices in groups of 10 were incubated for 24 h at 25°C with 4 ml of solution as indicated below. 1 ml  $M$  NaCl was added to the solutions before homogenizing in a Potter type homogenizer. The homogenates were left to stand for 1 h at room temperature before centrifuging (MSE bench centrifuge, 5 min, speed 10).  $\alpha$ -amylase activity in the supernatant was assayed at 25°C by the iodine-dextrin colour method of BRIGGS<sup>8</sup> and expressed in arbitrary units (AU) per 10 slices as described by DUFFUS<sup>9</sup>.

Prostaglandins  $E_1$ ,  $E_2$ ,  $A_1$  and  $F_{2\alpha}$  prepared by Dr. J. E. PIKE were made available by the Upjohn Company, Kalamazoo, Michigan 49001. Prostaglandin  $F_{2\alpha}$  supplied as the tromethamine salt was easily soluble in water. Solutions of prostaglandins  $E_1$ ,  $E_2$  and  $A_1$  were prepared by dissolving 1 mg of prostaglandin in 0.1 ml of 95% ethanol and making

up to 1.0 ml with sodium carbonate solution (0.2 mg/ml). The final pH was between 6 and 7.5.

The results show that prostaglandin  $F_{2\alpha}$  at a concentration of  $10^{-5}M$  can trigger  $\alpha$ -amylase release in barley endosperm slices. The effect is small compared to that of gibberellic acid at a concentration of  $10^{-5}M$  but is similar to that reported previously<sup>3</sup> for cyclic AMP at the same concentration. It does not appear to have an additive (or any) effect on the response to gibberellic acid. Prostaglandins  $E_1$ ,  $E_2$  and  $A_1$  had no effect, either alone or in combination with gibberellic acid. A control experiment showed that no inhibitory effect on gibberellic acid triggered  $\alpha$ -amylase release was observed with ethanolic sodium carbonate solution at the appropriate concentration. Polyunsaturated acids such as linolenic and linoleic acid, naturally occurring in mature barley seeds and thought to be precursors of prostaglandin in animals also had no effect.

The amount of  $\alpha$ -amylase released appears to be finite and does not increase with long incubation. It may be suggested, therefore, that prostaglandin  $F_{2\alpha}$  may bring about the release of a small amount of preformed  $\alpha$ -amylase, possibly through the mediation of cyclic AMP.

**Zusammenfassung.** Prostaglandin  $F_{2\alpha}$  kann die Freisetzung von  $\alpha$ -Amylase im Endosperm von Gerste (*Hordeum vulgare* L.).

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Relative activities of  $\alpha$ -amylase released by the action of gibberellic acid and prostaglandin  $F_{2\alpha}$

Addition	$\alpha$ -amylase activity in AU/10 slices
Gibberellic acid ( $10^{-5}M$ )	$0.34 \pm 0.08$ (14) <sup>a</sup>
Prostaglandin $F_{2\alpha}$ ( $10^{-5}M$ )	$0.020 \pm 0.013$ (14)
Distilled water	$< 0.0020$ (14)
Prostaglandin $E_1$ ( $10^{-5}M$ )	$< 0.0030$ (4)
Prostaglandin $E_2$ ( $10^{-5}M$ )	$< 0.0010$ (2)
Prostaglandin $A_1$ ( $10^{-5}M$ )	$< 0.0005$ (2)

<sup>a</sup> The number of experiments is given in brackets.

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## EFFECT OF GIBBERELIC ACID ON POLYPHENOL OXIDASE ACTIVITY IN DE-EMBRYONATED WHEAT AND BARLEY GRAINS

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### SUMMARY

De-embryonated grains of wheat and barley showed various responses when incubated with gibberellic acid ( $GA_3$ ). Amylase activity in the three cultivars studied showed similar responses to  $GA_3$  concentration and period of incubation. Changes in o-diphenolase and monophenolase activities of polyphenol oxidase (o-Diphenol: oxygen oxidoreductase, E.C.1.10.3.1) which occurred when de-embryonated grains were incubated in the absence of  $GA_3$ , were enhanced by the hormone. Activity was promoted in extracts from wheat half-grains, but inhibited in extracts from the two barley cultivars. Whereas monophenolase activity was detected primarily in extracts of the wheat aleurone layer, it was only detectable in the barley endosperm extracts. The inhibitory effect of  $GA_3$  on monophenolase activity in the barley cultivars is discussed.

### INTRODUCTION

Polyphenol oxidase activity has been studied in a number of plants and plant tissues in relation to plant disease development, wounding of plant tissue and hormonal regulation. No clear cut picture emerges as regards the first. In some cases the amount of detectable polyphenol oxidase activity increases with infection of the host plant by the pathogen (Jennings, Branneman and Zscheile, 1969; Kammen and Brouwer, 1964; Lovrekovich, Lovrekovich and Stahmann, 1967; Maxwell and Bateman, 1967), while in others the activity decreases (Farkas and Lovrekovich, 1965). Also, it is unclear whether these changes in enzyme activity are primary or secondary responses to disease development. In addition, mechanical wounding of plant tissue may result in increased polyphenol oxidase activity (Hulme and Rhodes, 1971; Hyodo and Uritani, 1966). Of the hormones tested for their effect in regulating polyphenol oxidase activity in plants, both ethylene (Abeles, 1972; Herrero and Hall, 1960; Stahmann, Clare and Woodbury, 1966) and indoleacetic acid (Stafford and Galston, 1970; Vernon and Straus, 1972) have been shown to cause an increase in enzyme activity in cotton leaves and tobacco pith cultures respectively. This by no means suggests a universal response or a direct cause and effect relationship. Gibberellic acid ( $GA_3$ ) has been shown to stimulate polyphenol oxidase (monophenolase) activity (Taneja and Sachar, 1974). As there are several other well-known enzyme systems which have been shown to be regulated by  $GA_3$  in cereal grains during germination, it was of interest to test the generality of the polyphenol oxidase response using de-embryonated grains. For this purpose one cultivar of spring wheat (Kleiber) and two cultivars of spring

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barley (Nakta and Golden Promise) were selected. It was necessary to dehusk only the Golden Promise since both Nakta and Kleiber lack an adhering husk at maturity. Amylase activity was also monitored in this study to serve as a basis for comparison with a known  $GA_3$  effect. No attempt was made to distinguish between alpha- and beta-amylase.

## MATERIALS AND METHODS

De-embryonated grains were surface-sterilized with 0.02% mercuric chloride for 10 min and rinsed ten times with distilled water. The half-grains (twenty) were placed in 5-cm Petri dishes with 3.5 ml of incubation medium containing the required concentrations of  $GA_3$ , incubated at 25°C in the dark, then removed for homogenizing; the residual incubation medium (RIM) was saved for enzyme assay. Controls were treated similarly, but in the absence of  $GA_3$ .

### *Enzyme extract*

A modified method of Taneja and Sachar (1974) was used where ten half-grains were homogenized with a chilled mortar and pestle using 4 ml of 0.05 M sodium phosphate buffer (pH 6.6) and centrifuged at 20,000 g for 15 min. The supernatant was used as the crude enzyme extract for all enzyme assays.

### *Amylase activity*

The assay mixture containing 1 ml of a soluble starch substrate (0.3 g/50 ml containing 1.5 ml of 1 M acetate buffer, pH 5.2), 0.4 ml enzyme and 0.1 ml water. The amount of enzyme was adjusted according to the activity of the extracts and the difference made up with water to maintain a constant volume of 1.5 ml in the assay mixture. A 0.1-ml aliquot was withdrawn after 2 and 4 min of incubation at 30°C and added to 5 ml of iodine solution (0.254 g  $I_2$  and 4 g KI/l). Absorbance was measured at 608 nm and activity expressed as change in absorbance per half-grain under the conditions of assay.

### *Polyphenol oxidase activity*

The assay of monophenolase and o-diphenolase activity was a modified method of Taneja and Sachar (1974). Monophenolase activity was assayed using 1 ml crude enzyme extract, 2 ml L-tyrosine (0.5 mg/ml) and 1 ml 0.05 M phosphate buffer, pH 6.6. The mixture was incubated at 37°C for 3 h and absorbance measured at 430 nm. For o-diphenolase activity the reaction involved 0.5 ml crude enzyme extract, 2 ml catechol (10 mg/ml) and 0.5 M phosphate buffer, pH 6.6. The change in absorbance was measured at 430 nm and was linear for at least 1 min. Reaction mixtures lacking substrate served as controls for both assays.

### *Nitrogen and o-diphenol determinations*

The nitrogen content of the dry de-embryonated grains was determined by the microKjeldahl procedure. For the o-diphenol determination, 5 g of dry, de-embryonated grains were extracted with 20 ml boiling 80% ethanol for 5 min. After decanting, the residue was re-extracted 3 times, each with 20 ml boiling 80% ethanol for 10 min. The extracts were combined, stored for at least 24 h at 4°C and then centrifuged for 20 min at 3,000 rev/min. The supernatant was made up to 100 ml with 80% ethanol and aliquots taken for determination of o-diphenol content by a modified procedure of Mapson, Swain and Tomalin.

(1963). To 10 ml of extract or a suitable dilution, was added 2 ml of 4% sodium molybdate in 50% ethanol. Another 10 ml sample was treated with 2 ml of 50% ethanol to serve as a control and the absorbance measured at 370 nm. When the efficiency of the extraction procedure was checked by adding a known amount of catechol to the sample before extraction, 100% recovery was attained. A standard curve was prepared using catechol and results are expressed as mg o-diphenol in terms of catechol equivalents. Absorbance was linear between 0.01 and 0.08 mg catechol/ml.

All experiments were conducted at least twice and unless stated otherwise the results presented represent the average of duplicate experiments.

## RESULTS

The effect of  $GA_3$  on amylase activity extracted from the de-embryonated grains is characteristic of this well-documented response. A slight enhancement of activity is observed at  $10^{-9}$  M  $GA_3$  with maximum response between  $10^{-6}$  and  $10^{-5}$  M  $GA_3$  (Fig. 1). Although the level of activity is quite similar in the three cereals, Golden Promise releases more than twice as much amylase to the incubation medium as the other two cultivars. The time course of activity is also quite similar among the three cereals tested (Fig. 2). The 24-h lag in Kleiber may reflect a slower uptake of  $GA_3$  due to its thicker pericarp. Little, if any, change in activity occurs in the absence of added  $GA_3$ .

The effect of  $GA_3$  on o-diphenolase activity is more complex and differences in response are seen between the wheat and the two barleys. Significant levels of enzyme activity are detectable at zero time in all three cereals with Nakta having the highest (Fig. 3). Activity in Kleiber increases over the whole of the 72-h incubation period being enhanced by  $GA_3$  after 48 h. In contrast, activity in Nakta decreased with time of incubation and the presence of

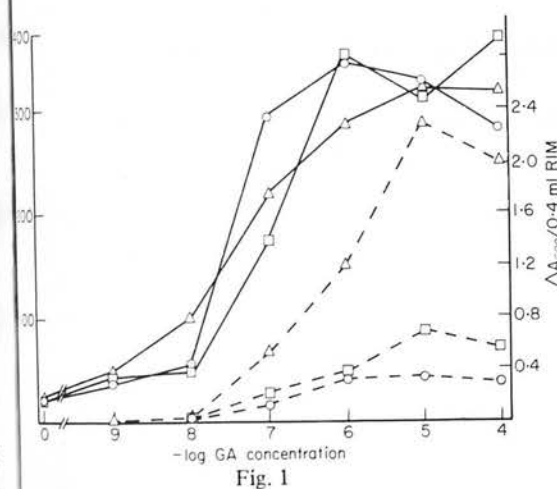


Fig. 1

Fig. 1. Effect of  $GA_3$  concentration on amylase activity ( $\Delta A_{608}$ ) extracted from de-embryonated grains incubated 48 h at 25°C and activity secreted to the residual incubation medium (RIM). (□) Kleiber; (○) Nakta; (Δ) Golden Promise. —, half-grain; ---, RIM.

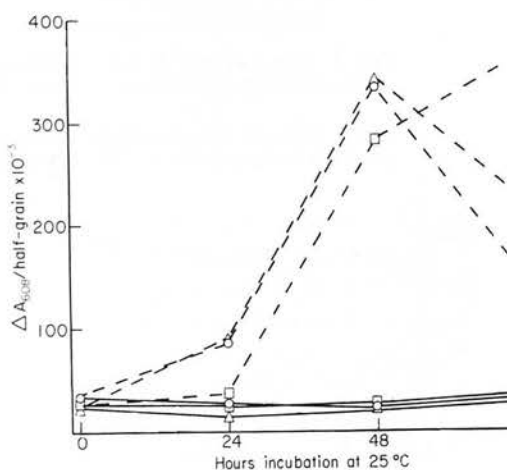


Fig. 2

Fig. 2. Effect of  $10^{-5}$  M  $GA_3$  on amylase activity ( $\Delta A_{608}$ ) extracted from de-embryonated grains. (□) Kleiber; (○) Nakta; (Δ) Golden Promise. —, Control; ---,  $10^{-5}$  MGA.

GA<sub>3</sub> resulted in even lower activity compared with the control after 48 and 72 h. The o-diphenolase activity of Golden Promise, which is lowest of the three grains at zero time, increased slightly during incubation as with Kleiber, but was reduced by GA<sub>3</sub> after 48 h. Whereas GA<sub>3</sub> had an inhibitory effect on o-diphenolase activity over the whole range of concentration tested on the barleys, activity appears to be stimulated in Kleiber between 10<sup>-7</sup> and 10<sup>-6</sup> M GA<sub>3</sub> (Fig. 4).

An inhibitory effect of GA<sub>3</sub> on monophenolase activity is also observed with the two barley cultivars, but not so with Kleiber (Fig. 5). Monophenolase activity is present at zero time in all three cereals, but in this case Kleiber has the highest initial level and this increases during incubation being enhanced slightly after 48 h incubation with GA<sub>3</sub> (Fig. 6). Activity remained fairly constant in the absence of GA<sub>3</sub>, but Golden Promise exhibited a marked reduction as early as 24 h when GA<sub>3</sub> was present.

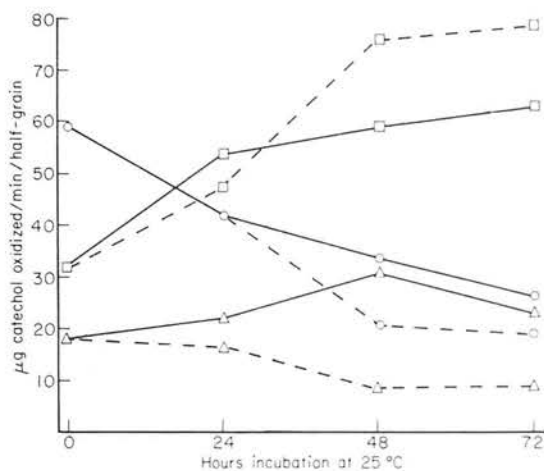


Fig. 3

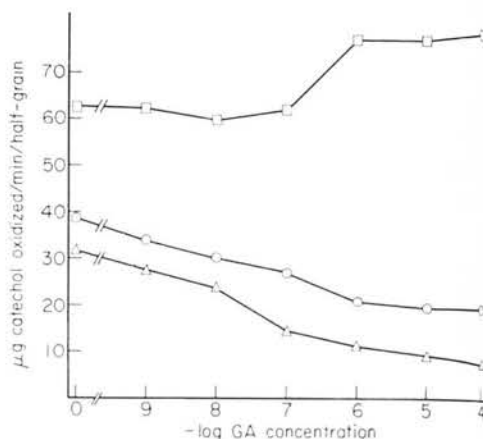


Fig. 4

Fig. 3. Effect of 10<sup>-5</sup> M GA<sub>3</sub> on o-diphenolase activity extracted from de-embryonated grains. (□) Kleiber; (○) Nakta; (△) Golden Promise. —, Control; ---, 10<sup>-5</sup> M GA.

Fig. 4. Effect of GA<sub>3</sub> concentration on o-diphenolase activity extracted from de-embryonated grains incubated 48 h at 25°C. (□) Kleiber; (○) Nakta; (△) Golden Promise.

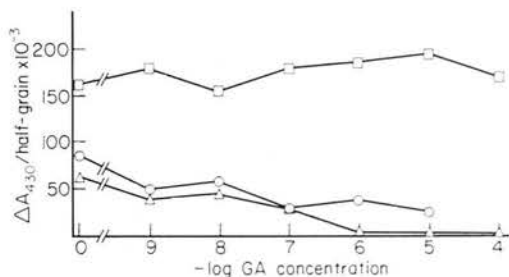


Fig. 5

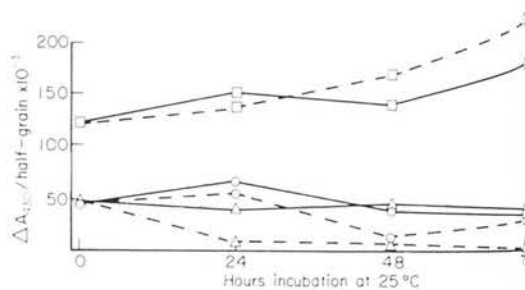


Fig. 6

Fig. 5. Effect of GA<sub>3</sub> concentration on monophenolase activity ( $\Delta A_{430}$ ) extracted from de-embryonated grains incubated 48 h at 25°C. (□) Kleiber; (○) Nakta; (△) Golden Promise.

Fig. 6. Effect of 10<sup>-5</sup> M GA<sub>3</sub> on monophenolase activity ( $\Delta A_{430}$ ) extracted from de-embryonated grains. (□) Kleiber; (○) Nakta; (△) Golden Promise. —, Control; ---, 10<sup>-5</sup> M GA.



In order to locate the enzyme activities under study, the aleurone layer and endosperm were separated after 24 h of incubation. In this case the aleurone layer also included the pericarp. The results indicate that o-diphenolase and amylase activities are present in extracts of both the aleurone and endosperm of all three cereal half-grains (Table 1). Monophenolase activity, however, was not detectable in aleurone extracts of the two barley cultivars but was detectable in the endosperm extracts. Kleiber, on the other hand, exhibited a high level of monophenolase activity in aleurone extracts and very little in the endosperm extracts.

The o-diphenol content of the dry half-grains does not appear to be correlated with the initial levels of o-diphenolase activity (Table 2). Kleiber, which develops the highest level of enzyme activity during incubation, contained the least o-diphenol in the dry grain, while Golden Promise, which maintained the lowest level of activity during incubation, was found to contain the highest o-diphenol content.

Table 1. *Enzyme activities in extracts of aleurone and endosperm separated from de-embryonated grains after 24 h of incubation.*

Cultivar	Aleurone			Endosperm		
	o-diphenolase $\mu\text{g}$ catechol oxidized/min half-grain	Monophenolase $\Delta A_{430}/$ half-grain ( $\times 10^{-3}$ )	Amylase $\Delta A_{608}/$ half-grain ( $\times 10^{-3}$ )	o-diphenolase $\mu\text{g}$ catechol oxidized/min half-grain	Monophenolase $\Delta A_{430}/$ half-grain ( $\times 10^{-3}$ )	Amylase $\Delta A_{608}/$ half-grain ( $\times 10^{-3}$ )
Kleiber	75	177	19	24	10	17
Nakta	31	0	20	11	31	13
Golden Promise	14	0	13	26	10	20

Table 2. *Nitrogen and o-diphenol content of dry, de-embryonated grains*

Cultivar	Nitrogen (mg N/half-grain)	o-diphenol (as $\mu\text{g}$ catechol/half-grain)
Kleiber	0.577	13.9
Nakta	0.470	37.8
Golden Promise	0.308	83.4

## DISCUSSION

Amylase activity extracted from the three cereals studied exhibited a very similar response to  $\text{GA}_3$  concentration and time of incubation; little change occurred when the de-embryonated grains were incubated in the absence of the hormone (Figs. 1 and 2). The activities of o-diphenolase and monophenolase, on the other hand, varied widely among the three grains even at zero time and changes in activity occurred during incubation in the absence of  $\text{GA}_3$  (Figs. 3 and 6). For the most part,  $\text{GA}_3$  only enhanced those changes in activity which occurred during incubation and in the case of the two barley cultivars, lowered the level of detectable activity extracted from the half-grains (Figs. 4 and 5). In contrast to these results, Taneja and Sachar (1974) reported an inhibition of o-diphenolase and stimulation of monophenolase activities by  $\text{GA}_3$  with the wheat cultivar Sharbati Sonora. These differences in response to  $\text{GA}_3$  suggest that the regulation of o-diphenolase and monophenolase in incubated, de-embryonated cereal grains is different from that shown for alpha-amylase and protease (Chrispeels and Varner, 1967; Glasziou, 1969; Jacobsen and Varner, 1967; Paleg, 1960).

The inhibitory effect of  $GA_3$  on o-diphenolase and monophenolase activities when the two barley cultivars were incubated with the hormone might possibly be the result of  $GA_3$ -stimulated protease activity. A similar interpretation was made in the case of nitrate-induced nitrate reductase activity of barley aleurone layers in which  $GA_3$  was shown to have an inhibitory effect (Ferrari and Varner, 1969). It was also shown that nitrate had no effect on  $GA_3$ -stimulated amylase activity.

The difference in nitrogen contents of the three cereal grains suggests that Kleiber has a higher protein content than the two barley cultivars (Table 2). This may serve as a suitable substrate for protease activity, thus diverting the enzyme and thereby sparing the polyphenol oxidases from proteolytic attack. The lower protein contents of the two barley cultivars may not be as effective in this regard and thus polyphenol oxidase activity is lowered through  $GA_3$ -stimulated proteolysis.

The location of enzyme activities within the de-embryonated grains appears to differ between the three cereal grains. Taneja and Sachar (1974) located monophenolase activity in the aleurone layer of the wheat cultivar used in their study and this agrees with our results for Kleiber (Table 1). However, in the present work, monophenolase activity was detectable only in the endosperm extracts of the two barley cultivars tested. Thus, if monophenolase is subject to proteolytic attack and  $GA_3$  promotes protease activity which is then secreted to the endosperm, then monophenolase activity in Nakta and Golden Promise might be expected to decrease with time of incubation with  $GA_3$  and increasing  $GA_3$  concentration (Figs. 5 and 6). Both o-diphenolase and amylase activities, on the other hand, were found in extracts of the aleurone and endosperm of all three grains. Although it is possible that some movement of enzyme activity between the two tissues may have occurred at the time of tissue separation (24 h of incubation) and that the tissues were not completely separated, it is felt that the localization of activity observed represents fairly closely the origins of the enzymes in question.

The evidence presented suggests that there are varietal differences in the response of o-diphenolase and monophenolase activities to  $GA_3$  and that the hormone in part serves to enforce changes in activity which take place in its absence. Further studies on the effect of  $GA_3$  on o-diphenolase are being pursued and will be reported in another paper.

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## CONTROL OF POLYPHENOL OXIDASE (O-DIPHENOLASE) IN DE-EMBRYONATED BARLEY AND WHEAT GRAINS

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### SUMMARY

Different control mechanisms were shown to exist which affected the level of o-diphenolase (o-Diphenol: Oxygen oxidoreductase, E.C.1.10.3.1) activity in extracts of two barley cultivars and one wheat cultivar. Both the increase in o-diphenolase activity which occurs in Kleiber wheat during half-grain incubation and the activity promoted by gibberellic acid ( $GA_3$ ) were dependent on protein synthesis and could be inhibited by cycloheximide and abscisic acid respectively. Incubation with  $GA_3$  lowered o-diphenolase activity in the barley, Nakta, which was not due to  $GA_3$  promoted protease activity. The role of protein synthesis and protease activity in controlling the levels of detectable o-diphenolase activity is discussed.  $GA_3$  caused a direct, stimulatory effect on o-diphenolase activity extracted from both Kleiber and Nakta when the hormone was added to the assay mixture.

### INTRODUCTION

Previous work (Jennings and Duffus, 1977) has shown that o-diphenolase activity extracted from de-embryonated wheat and barley grains changes during incubation. The cultivars differed in their response to gibberellic acid ( $GA_3$ ) which enhanced activity when incubated with wheat half-grains, but inhibited activity in barley half-grains. In contrast, Taneja and Sachar (1974a) have reported that  $GA_3$  inhibits o-diphenolase activity in de-embryonated wheat grains. The purpose of the present work is to investigate further the effect of  $GA_3$  on the regulation of o-diphenolase activity in de-embryonated grains of barley and wheat and to compare the response with  $GA_3$  controlled amylase activity.

### MATERIALS AND METHODS

De-embryonated grains of the spring wheat, Kleiber, and the spring barleys, Nakta and Golden Promise, were surface sterilized with 0.02% mercuric chloride for 10 min and rinsed 10 times with distilled water. The half grains were incubated at 25°C in 5-cm Petri dishes in 3.5 ml of incubation medium, containing the appropriate concentrations of  $GA_3$ , phosphate buffer, abscisic acid or cycloheximide. The extraction and assay of o-diphenolase and amylase was as previously described (Jennings and Duffus, 1977). All experiments were conducted at least twice and results represent the averages of duplicate experiments.

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## RESULTS

Table 1 presents the results of an experiment in which half-grains were incubated in either distilled water or 0.05 M sodium phosphate buffer pH 6.6, each  $\pm 10^{-5}$  M  $GA_3$ . Phosphate buffer was also used for all enzyme extractions as previously described (Jennings and Duffus, 1977). In addition, either phosphate buffer or  $10^{-5}$  M  $GA_3$  was added to the enzyme assay mixture in an attempt to resolve whether or not the  $GA_3$  effect was a direct one on the enzyme itself. In the case of the Kleiber and Nakta two distinct effects of  $GA_3$  are seen depending on whether or not the half-grains were incubated with the hormone and whether or not  $GA_3$  was included in the assay mixture. Extracts from Kleiber exhibited increased o-diphenolase activity when half-grains were incubated with the hormone and this effect was eliminated when phosphate buffer was included in the incubation medium. Regardless of the conditions of incubation, all Kleiber extracts showed enhanced activity when  $GA_3$  was added

Table 1. *The effect of adding gibberellic acid ( $GA_3$ ) to buffered or unbuffered assay mixture on o-diphenolase activity extracted from de-embryonated grains incubated 48h*

Cultivar	Additions to assay mixture	o-diphenolase activity ( $\mu$ g catechol oxidized/min/half grain)			
		Incubation medium		Phosphate buffer pH 6.6	
		Control			
		— $GA_3$	+ $GA_3$	— $GA_3$	+ $GA_3$
Kleiber	Phosphate buffer pH 6.6	61	69	34	34
	$GA_3$ $10^{-5}$ M	68	75	49	45
Nakta	Phosphate buffer pH 6.6	35	20	42	25
	$GA_3$ $10^{-5}$ M	47	26	48	35
Golden Promise	Phosphate buffer pH 6.6	25	9	17	12
	$GA_3$ $10^{-5}$ M	26	8	19	12

to the assay mixture. This same enhancement of activity was noted when  $GA_3$  was added to the assay mixture of enzyme extracts from Nakta. However, as previously reported (Jennings and Duffus, 1977)  $GA_3$  has an inhibitory effect on o-diphenolase activity in Nakta when the half-grains are incubated with the hormone and in the experiment reported here this effect persisted whether or not phosphate buffer was present in the incubation medium. Golden Promise responded similarly to Nakta in this last respect; however, addition of  $GA_3$  to the assay mixture of extracts from Golden Promise did not stimulate o-diphenolase activity.

Phosphate buffer was also shown to have an inhibitory effect on  $GA_3$  promoted amylase activity of all three cultivars when included in the incubation medium (Table 2). Activity was reduced by 30% with Golden Promise and over 80% in Kleiber. The  $GA_3$  controlled secretion of amylase was also affected by inclusion of buffer in the incubation medium and appeared to be more than a reflection of the buffer effect on the level of activity in the half-grains.

It has been shown that abscisic acid (ABA) is able to inhibit the  $GA_3$  promotion of  $\alpha$ -amylase and protease in barley aleurone layers (Chrispeels and Varner, 1967; Jacobsen and Varner, 1967). In order to determine whether the  $GA_3$  effect on o-diphenolase activity was similar to the  $GA_3$  control of  $\alpha$ -amylase activity in barley, an experiment was set up to study the interaction of  $GA_3$  and ABA on the activities of these two enzyme systems. Table 3 shows the inhibitory effect of ABA on  $GA_3$ -stimulated amylase activity in extracts from both Kleiber and Nakta. The  $GA_3$ -mediated secretion of amylase activity was also inhibited

by ABA. The stimulation of o-diphenolase activity by incubation of Kleiber half-grains with  $GA_3$  was inhibited by  $10^{-5}$  M but not by  $10^{-7}$  M ABA. The o-diphenolase activity extracted from Nakta, which is lowered by incubation with  $GA_3$ , was not restored to the control level by co-incubation with  $10^{-5}$  M ABA.

To investigate the role of protein synthesis in  $GA_3$ -induced changes of o-diphenolase activity, half-grains were incubated with either  $GA_3$ , cycloheximide, or both simultaneously, and the effects on o-diphenolase and amylase activities determined after 48 h incubation. The results (Table 4) show that cycloheximide reduced the level of  $GA_3$ -promoted amylase activity, presumably through inhibition of protein synthesis although cycloheximide has been shown to have other effects including an effect on respiration (Ellis and MacDonald, 1970). However, unlike the inhibitory effect of ABA on  $GA_3$ -mediated secretion of amylase activity

Table 2. Effect of buffered and unbuffered gibberellic acid ( $GA_3$ ) on amylase activity extracted from de-embryonated grains incubated 48 h and on amylase activity secreted to the residual incubation medium (RIM)

Incubation Medium	Amylase $\Delta A_{608}/\text{half-grain}$ ( $\times 10^{-3}$ )			Amylase in RIM $\Delta A_{608}/0.4 \text{ ml RIM}$		
	Kleiber	Nakta	Golden Promise	Kleiber	Nakta	Golden Promise
Control	24	24	20	0.041	0.001	0
Phosphate buffer pH 6.6	29	29	30	0.092	0.010	0
$GA_3$ $10^{-5}$ M in						
Phosphate buffer pH 6.6	52	137	201	0.093	0.015	0.046
$GA_3$ $10^{-5}$ M	305	261	299	0.646	0.205	2.184

Table 3. Effects of gibberellic acid ( $GA_3$ ) and abscisic acid (ABA) on enzyme activities extracted from incubated de-embryonated Nakta and Kleiber half-grains and on enzyme activity secreted to the residual incubation medium (RIM)

Incubation medium	o-diphenolase activity $\mu\text{g}$ catechol oxidized/ min/half-grain		Amylase activity $\Delta A_{608}/\text{half-grain}$ ( $\times 10^{-3}$ )		RIM amylase $\Delta A_{608}/0.4 \text{ ml RIM}$	
	Kleiber	Nakta	Kleiber	Nakta	Kleiber	Nakta
Control	55	37	23	24	0.103	0
$GA_3$ $10^{-6}$ M	77	21	327	345	0.580	0.330
ABA $10^{-5}$ M	54	41	26	27	0.073	0
$GA_3$ $10^{-6}$ M + ABA $10^{-5}$ M	56	20	162	169	0.267	0.015
$GA_3$ $10^{-6}$ M + ABA $10^{-7}$ M	76	28	296	292	0.350	0.035

Table 4. Effects of gibberellic acid ( $GA_3$ ) and cycloheximide (CH) on enzyme activities extracted from de-embryonated Nakta and Kleiber half-grains after 48 h incubation and on enzyme activity secreted to the residual incubation medium (RIM).

Incubation Medium	o-diphenolase activity $\mu\text{g}$ catechol oxidized/ min/half-grain		Amylase activity $\Delta A_{608}/\text{half-grain}$ ( $\times 10^{-3}$ )		RIM amylase $\Delta A_{608}/0.4 \text{ ml RIM}$	
	Kleiber	Nakta	Kleiber	Nakta	Kleiber	Nakta
Control	62	39	24	24	0.041	0.001
$GA_3$ $10^{-5}$ M	69	29	305	261	0.646	0.205
CH $1 \mu\text{g}/\text{ml}$	42	31	22	32	0	0
$GA_3$ $10^{-5}$ M + CH $1 \mu\text{g}/\text{ml}$	34	31	34	126	0.130	0.120



to the incubation medium, cycloheximide did not appear to have any distinct effect on amylase secretion, and the degree of inhibition of secretion was approximately proportional to the amount of inhibition of  $GA_3$ -promoted activity extracted from the incubated half-grains. Whereas cycloheximide had no effect on amylase activity extracted from half-grains incubated in the absence of  $GA_3$ , the o-diphenolase activities of both Kleiber and Nakta were reduced under these conditions suggesting that  $GA_3$ -independent protein synthesis may be involved in determining the level of o-diphenolase activity extracted from incubated half-grains.

## DISCUSSION

Although the magnitude of the response of o-diphenolase activity to  $GA_3$  is small compared with that of amylase activity, the results presented here show two distinct effects of the hormone on o-diphenolase. Previously, we have shown that o-diphenolase activity in Kleiber half-grains increased during incubation even in the absence of  $GA_3$  (Jennings and Duffus, 1977). This increase appears to be dependent on protein synthesis as cycloheximide at  $1 \mu\text{g/ml}$  eliminated it during a 48 h incubation of the half-grains as compared with the control (Table 4). Whereas no effect was observed on amylase activity under these conditions, when both cycloheximide and  $GA_3$  were included in the incubation medium there was an inhibition of both amylase and o-diphenolase activities.

The response of o-diphenolase to  $GA_3$  reported here may be similar to that of ribonuclease and  $\beta$ -1, 3-glucanase activities which have been reported to increase *de novo* during incubation (Bennett and Chrispeels, 1972) and are only slightly enhanced by  $GA_3$  (Chrispeels and Varner, 1967; Jones, 1971). The enhancement of o-diphenolase activity may be the result of specific isozymic changes which have been shown to occur during germination of wheat grains (Taneja and Sachar, 1974b).

Regardless of whether or not  $GA_3$  was included in the incubation medium, o-diphenolase activity was considerably lower when half-grains were incubated in the presence of buffer (Table 1). The level of activity observed after 48 h incubation with buffer was similar to that measured when incubated with cycloheximide (Table 4) or at zero time during a time course incubation (Jennings and Duffus, 1977). Thus the inhibition of o-diphenolase when Kleiber half-grains were incubated with phosphate buffer may be the result of inhibition of  $GA_3$ -independent protein synthesis. A similar inhibition is seen in the case of  $GA_3$ -promoted amylase activity (Table 2) which is known to involve *de novo* synthesis of the enzyme (Jones, 1973; Filner and Varner, 1967; Varner and Ram Chandra, 1964).

The stimulatory effect of  $GA_3$  on o-diphenolase activity extracted from Kleiber half-grains when added to the assay mixture is a separate and distinct response occurring even when an increase in activity has been inhibited by incubation of the half-grains with phosphate buffer (Table 1). As far as we are aware this is the first report of a direct stimulatory effect of  $GA_3$  on enzyme activity.

The results with Nakta present a different picture in terms of hormonal effects and changes in o-diphenolase activity. As shown previously, enzyme activity is comparatively high at zero time during a time course incubation, then subsequently declines (Jennings and Duffus, 1977). Incubation with  $GA_3$  lowers the level of extractable activity even further in contrast to the promotive effect with Kleiber. Ferrari and Varner (1969) reported that  $GA_3$  inhibited nitrate-induced nitrate reductase activity in barley aleurone layers, while nitrate had no effect on  $GA_3$ -promoted amylase activity. They suggested that the inhibition of

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nitrate reductase by  $GA_3$  may have occurred due to  $GA_3$ -promoted protease activity. However, since co-incubation with ABA, an inhibitor of  $GA_3$ -promoted protease activity (Jacobsen and Varner, 1977), does not relieve the inhibition of o-diphenolase activity in extracts of Nakta incubated with  $GA_3$  (Table 3) it is likely that  $GA_3$  may be operating via some other mechanism. Further evidence that protein synthesis may not be involved in this particular  $GA_3$  response comes from the results with cycloheximide where, either alone or in combination with  $GA_3$  during incubation, it had little effect in either further reducing o-diphenolase activity or preventing the lowering of activity during a 48 h incubation (Table 4). Protease activity has been detected in both barley aleurone layers and endosperm after 24 h of incubation even in the absence of  $GA_3$  (Jacobsen and Varner, 1967). Whereas  $GA_3$  caused a ten-fold increase in activity in the aleurone it had no effect on endosperm protease. It is possible, then, that endosperm protease in Nakta was responsible for the decline in o-diphenolase activity during incubation. Inclusion of  $GA_3$  in the incubation may further lower activity by directly stimulating protease activity. The higher o-diphenolase activity observed when Nakta half-grains were incubated with buffer, whether or not  $GA_3$  was present, might then be explained as due to less favourable conditions for protease activity, thus providing for a slower proteolysis of protein and destruction of o-diphenolase. Sundblom and Mikola (1972) found several proteases in barley aleurone and reported that a main component was a labile sulfhydryl enzyme with a pH optimum of 3.9. Thus the pH 6.6 phosphate buffer used in our incubation medium may have inhibited protease activity in Nakta half-grains.

Golden Promise responded similarly to Nakta in terms of effects on o-diphenolase activity when incubated with  $GA_3$ . However, unlike Nakta, o-diphenolase activity did not decrease during incubation but rather increased slightly when half-grains were incubated in the absence of  $GA_3$ . Therefore, if phosphate buffer inhibits protein synthesis, then o-diphenolase activity extracted from incubated Golden Promise half-grains would be lower when incubated in the presence of buffer. This in fact did occur (Table 1). Similarly, if  $GA_3$  directly stimulated a pre-formed protease, then incubation with  $GA_3$  would lead to lower levels of extractable o-diphenolase (Table 1).

It is clear that genetic differences exist between cereal cultivars which determine the control mechanisms for regulating o-diphenolase activity; however, different mechanisms appear to exist in the three cultivars studied. While  $GA_3$  stimulates activity by a direct effect on the enzyme it is possible that  $GA_3$  may also control specific isozymic forms of o-diphenolase. Additional studies are needed on endosperm proteases to elucidate differences between cultivars and *in situ* functions.

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### GROUP 3

#### Related Studies

BBA 43178

**Intracellular localisation of phosphoglycollate phosphatase and glyoxalate reductase**

The mechanism of formation of glycollic acid in photosynthesis is as yet uncertain. It is most probably derived from a sugar phosphate of the photosynthetic carbon reduction cycle<sup>1,2</sup> although some workers have suggested that it is derived *de novo* from an as yet unknown carboxylation reaction<sup>3,4</sup>.

A possible precursor of glycollate is phosphoglycollic acid and a specific phosphatase (phosphoglycollate phosphohydrolase, EC 3.1.3.18) has been described by RICHARDSON AND TOLBERT<sup>5</sup>. Whilst this enzyme has been reported to be predominantly cytoplasmic, its site of action has not been rigorously established.

A glyoxalate reductase (glycollate:NADP<sup>+</sup> oxidoreductase) which catalysed the reduction of glyoxalate to glycollate by NADPH has been described and partially purified by ZELITCH AND GOTTO<sup>6</sup>. Apart from the observation by ZELITCH AND BARBER<sup>7</sup> that NAD glyoxalate reductase activity was present in dilute suspensions of chloroplasts, its site of action has not been investigated in higher plants.

The distribution within the cell of phosphoglycollate phosphatase and glyoxalate reductase has been determined and the results discussed in relation to glycollate synthesis and metabolism.

Chloroplasts were isolated from freeze-dried spinach leaves on a non-aqueous density gradient as described by BIRD, PORTER AND STOCKING<sup>8</sup>. A suitable discontinuous density gradient which gives high yields of chloroplasts with minimal cytoplasmic contamination consisted of 15 ml of density 1.36 g/cm<sup>3</sup> containing 50 mg of homogenised and filtered leaf material followed by an equal volume of density 1.34 g/cm<sup>3</sup> and 2 ml density 1.32 g/cm<sup>3</sup>. The solvents used in making up the different densities were carbon tetrachloride and hexane. The yield of chloroplasts on a dry weight basis was between 4-6 % of the initial freeze-dried material. More than 55 % of the total cell protein remained with the chloroplasts. No mitochondrial activity (for which fumarase and succinate dehydrogenase were considered as characteristic) was detected in these preparations. Under the light microscope, cell debris could not be seen in these preparations. The chloroplasts appear to have the stroma matrix intact<sup>9,10</sup>. Chlorophyll was measured by the method of ARNON<sup>11</sup> and protein by the method of LOWRY *et al.*<sup>12</sup>.

The distribution of enzyme between the chloroplast and the cytoplasm was calculated by the method of HEBER<sup>13</sup>. From the specific activities of chloroplasts, intact tissue and chloroplast depleted tissue, it is possible to obtain a value for the specific activity of pure cytoplasm. The percentage of the total enzyme activity of the homogenate present in the chloroplast fraction could then be calculated. The intracellular distribution of phosphoglycollic acid phosphatase and glyoxalate reductase is shown in Tables I and II. Between 89-97 % of the total cell phosphoglycollate phosphatase and 86-98 % of the whole cell glyoxalate reductase were found to be localised in the chloroplasts.

Neither enzyme could be detected in chloroplasts from a glycerol gradient (prepared as described by LEECH<sup>14</sup> and JAMES AND DAS<sup>15</sup>). Assuming that the loss of activity was not due to solvent inactivation it was concluded that both enzymes were localised in the soluble stroma of the chloroplasts rather than in the lamellae.

The enzyme localisation is consistent with the view that glycollate is synthesised in a chloroplast sited reaction from both phosphoglycollate and glyoxalate. The chloroplast is also most probably the site of phosphoglycollate synthesis. KEARNEY AND TOLBERT<sup>16</sup> found phosphoglycollate as well as glycollate in the supernatant fluid

TABLE I

## ACTIVITY AND DISTRIBUTION OF PHOSPHOGLYCOLLATE PHOSPHATASE

Reaction mixtures contained in a final volume of 0.31 ml the following components in  $\mu$ moles:  $Mg^{2+}$ , 0.3; Tris buffer (pH 7.5), 30; enzyme suspension and water. The reaction was started by the addition of 2  $\mu$ moles of phosphoglycollate and the amount of inorganic phosphate released was measured colorimetrically by the method of HURST<sup>19</sup>.

	Expt. No.	
	1	2
Chloroplast protein in total protein (%)	73.5	56.0
Specific activity ( $\mu$ moles $P_i$ /min per mg protein)		
in chloroplasts	3.2*	4.46
in intact tissue	3.53**	5.3
in chloroplast depleted tissue	2.64	2.63
in cytoplasm	2.00	1.73
	1.08*	0.3
	0.202**	0.057
Yield of isolated chloroplasts		
(% of total chlorophyll content of tissue)	32.5	45.0
Total enzyme present in chloroplasts (%)	89.0*	94.5
	96.8**	98.7

\* Results uncorrected for cytoplasmic contamination.

\*\* Corrected values assuming that not more than 10% of the total cytoplasmic activity is associated with the chloroplasts.

TABLE II

## ACTIVITY AND DISTRIBUTION OF NADPH GLYOXALATE REDUCTASE

Enzyme activity was measured according to ZELITCH AND GOTTO<sup>8</sup>.

	Expt. No.	
	1	2
Chloroplast protein in total protein (%)	71.6	72.0
Specific activity (m $\mu$ moles NADPH/min per mg protein)		
in chloroplasts	21.53*	6.0
in intact tissue	24.5**	6.86
in chloroplast depleted tissue	10.3	4.5
in cytoplasm	14.4	3.63
	3.11*	0.64
	6.2**	0.204
Yield of isolated chloroplasts		
(% of total chlorophyll content of tissue)	49.7	35.0
Total enzyme present in chloroplasts (%)	94.0*	96.0
	86.0**	98.2

\* Results uncorrected for cytoplasmic contamination.

\*\* Corrected values assuming that not more than 10% of the total cytoplasmic activity is associated with the chloroplasts.



after separation of chloroplasts. It is probable that phosphoglycollate and glycollate are both derived from a sugar diphosphate of the photosynthetic carbon reduction cycle.

The function of glyoxalate reductase in chloroplasts is as yet unknown. It may be involved in the synthesis of amino acids within the chloroplast. Both SMITH, BASSHAM AND KIRK<sup>17</sup> and STEWARD, BIDWELL AND YEMM<sup>18</sup> have found that although glycine was a frequent constituent of plant leaf protein, the free acid was synthesised very slowly. It was suggested that the glycine residues in chloroplast protein were derived from the cytoplasm—perhaps from a 2-carbon compound such as glycollate. KEARNEY AND TOLBERT<sup>16</sup> have demonstrated glycine and glycollate formation in chloroplasts from glyoxalate.

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## GLYCOLLATE METABOLISM IN PHOTOSYNTHESISING TISSUE

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## SUMMARY

1. Glycollate oxidase (glycollate: oxygen oxidoreductase, EC 1.1.3.1) was not detected in chloroplasts prepared by the method of WALKER, in non-aqueous chloroplasts or in chloroplast lamellae.

2. The oxidase activity associated with some chloroplast preparations was shown to be due to adsorption from the supernatant solution on to the chloroplasts and to cytoplasmic contamination.

3. The results from experiments *in vitro* were confirmed *in vivo* by feeding of [ $^{14}\text{C}$ ]glycollate to spinach leaf tips. Only a small fraction of the counts taken up was found in the chloroplasts and glycollate itself was not metabolised by non-aqueously isolated chloroplasts.

4. The possible function of glycollate in carbon and hydrogen transport is discussed.

## INTRODUCTION

Glycollic acid formation has been reported during photosynthesis in unicellular algae<sup>1-5</sup>, in higher plants<sup>5,6</sup> and in isolated chloroplasts<sup>22</sup>. While the almost stoichiometric conversion of  $\text{CO}_2$  to glycollate at low  $\text{CO}_2$  partial pressures reported by WARBURG AND KRIPPAHL<sup>1</sup> is not generally observed, there is little doubt that a large fraction of newly fixed  $\text{CO}_2$  may be metabolised *via* glycollate and its derivatives<sup>5,7</sup>. A pathway from glycollate to sucrose *via* glycine and serine has been demonstrated in a variety of tissues<sup>8-13</sup>. This pathway can be blocked *in vivo* using an inhibitor of glycollate oxidase, when glycollate accumulates in large amounts<sup>14</sup>.

Both phosphoglycollate phosphatase (phosphoglycollate phosphohydrolase, EC 3.1.3.18) and glyoxalate reductase (glycollate: NADP oxidoreductase)—two enzymes probably involved in glycollate synthesis—are present almost exclusively in the chloroplast<sup>15</sup>. Little, however, is known of the intracellular site of action of glycollate oxidase (glycollate: oxygen oxidoreductase, EC 1.1.3.1). Activity has been attributed to the chloroplast<sup>16,17</sup>, the mitochondrion<sup>18</sup> and to the soluble supernatant<sup>19,20</sup>.

Much of the reported variation in results has been due to the use of contami-

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

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nated chloroplast preparations. It has been shown, for example, that after centrifugation at  $1000 \times g$  the sediments not only contain chloroplasts but also biochemically active mitochondria<sup>21</sup>.

There is much speculation regarding the role of glycollate in intracellular metabolism. In the present work glycollate oxidase has been assayed in chloroplasts isolated by several different techniques. The significance of the results is discussed in relation to [<sup>14</sup>C]glycollate feeding experiments *in vivo*.

#### METHODS

Spinach was the major source of plant material. In spring and summer it was possible to use outdoor-grown plants. In winter they were grown in the greenhouse under conditions of normal winter daylength. Only completely healthy young leaves were used, being removed from the plant not more than 15 min before the beginning of an experiment. The midribs were cut out and the leaves washed with distilled water, dried between blotting paper sheets and placed in a cold room at 4° before use.

Chloroplasts were extracted according to the method of ARNON, WHATLEY AND ALLEN<sup>22</sup>. The grinding medium used was 0.1 M phosphate buffer (pH 8.0) containing 0.01 M sodium isoascorbate and 0.35 M NaCl. The pellet from the first centrifugation at  $1000 \times g$  was washed by suspension in 0.35 M NaCl containing 0.01 M sodium isoascorbate. After the second centrifugation the pellet was resuspended in the same medium. Such a preparation was referred to as 'Arnon type' chloroplasts.

An alternative method of chloroplast isolation used was that due to WALKER<sup>23,24</sup>. 0.4 M sucrose in both the grinding medium and the resuspending medium was replaced by 0.35 M NaCl. Chloroplasts prepared by this method were termed 'Walker type' chloroplasts. It was shown that whilst they did not preserve their structure as long as those prepared in sucrose or sorbitol, the preparation had a majority of plastids with their outside membranes intact immediately after preparation.

Chloroplast lamellae were prepared according to LEECH<sup>25</sup> after the method of JAMES AND DAS<sup>26</sup>. The final pellet was suspended in 0.006 M Tris-HCl buffer (pH 7.5) containing 0.3 M sucrose.

Chloroplasts were isolated non-aqueously by the method of BIRD, PORTER AND STOCKING<sup>27</sup>. 100 mg of freeze-dried spinach leaves were homogenised in 15 ml of a carbon tetrachloride-hexane mixture,  $d = 1.36 \text{ g/cm}^3$ . After filtration, and readjustment of the volume to 15 ml the suspension was transferred to a centrifuge tube and an equal volume of density 1.34 was layered on top. A further 2-ml volume of density 1.32 completed the gradient. After centrifugation the top layer containing the chloroplasts was removed, diluted with an equal volume of hexane and spun down at  $3000 \times g$ . The last traces of organic solvent were removed by evacuating the tubes in a desiccator on a water pump for 10 min. The chloroplasts appeared after this final operation as a bright green powder. The yield on a weight basis was between 4-6 % of the initial freeze-dried material. Resuspending media were as indicated in the RESULTS section. Chlorophyll was determined by the method of ARNON<sup>28</sup> and protein by that of LOWRY *et al.*<sup>29</sup>.

Glycollate oxidase was partially purified by the method of ZELITCH AND OCHOA<sup>30</sup>. The fraction from the second ethanol precipitation was used as a source of

crude enzyme and had a specific activity of 1.3  $\mu$ moles glycollate oxidised per mg protein per h.

Fumarase (L-malate hydro-lyase, EC 4.2.1.2) activity was measured by the increase in absorbance at 240 m $\mu$  on addition of malate (PIERPOINT<sup>19</sup>).

[1-<sup>14</sup>C]Glycollate was fed in the light *via* the petiole of spinach leaf tips as described by MIFLIN, MARKER AND WHITTINGHAM<sup>34</sup>. After photosynthesis for different periods of time, the leaves were removed individually with tweezers and the cut edge washed and dried. The leaves were then cut into thin strips and dropped into a flask precooled to -195° in liquid N<sub>2</sub>. The time between removal from the trough and freezing was never more than 20 sec.

The non-aqueous technique for the isolation of chloroplasts required a minimum of 100 mg freeze-dried material. This was equivalent to about 1 g fresh weight. Thus for each separate isolation procedure at least 6 leaf tips were fed.

When sufficient leaf material had been obtained the flask was transferred from the liquid N<sub>2</sub> to an acetone-solid CO<sub>2</sub> mixture at -79°. The contents were later freeze-dried and the chloroplasts isolated as described above. The chloroplast and chloroplast-depleted cytoplasm fractions were extracted twice in boiling 90 % ethanol. The insoluble residue was further extracted in 20 % ethanol and the combined supernatants were concentrated in a rotary evaporator at a temperature not greater than 35°. Under these conditions the distillate did not become radioactive. The residue, after vacuum desiccation was taken up in a known volume of 50 % ethanol. A fraction was counted under an end window Geiger-Müller tube to give the total counts in the soluble fraction and a fraction was retained for chromatography. The original insoluble residue was transferred to a small tube, dried down in a vacuum desiccator and suspended in a known volume of 50 % ethanol. Aliquots were dried down and counted as above.

The distribution of label in the soluble fraction was determined using the method of 2-dimensional chromatography as described by PRITCHARD, GRIFFIN AND WHITTINGHAM<sup>35</sup>.

## RESULTS

Some glycollic acid oxidase was present in Arnon type chloroplasts but none was found in those prepared by the method of WALKER<sup>23,24</sup> (Fig. 1).

Glycollate oxidase activity was removed from Arnon type chloroplasts after passage down a glycerol-sucrose density gradient. Electron micrographs indicate that such 'purified' preparations are devoid of external membranes and stroma and that there is little mitochondrial contamination<sup>25</sup>. Thus cytoplasmic material originally associated with the chloroplasts is removed after centrifugation on a glycerol gradient. Were the activity to remain in the chloroplasts after 'purification' it would almost certainly be associated with the lamellae; on the other hand absence of activity can only eliminate a lamellar but not a stromal or outer membrane site of action. Results are shown in Fig. 2. It was further shown that glycollate oxidase was readily absorbed on to chloroplasts during their isolation from a tissue homogenate. Of glycollate oxidase added to the grinding medium about 20 % became adsorbed to the chloroplast fraction (Table I). About one fourth to one third of the total original chloroplastic glycollate oxidase was lost in the first washing. A further washing removed

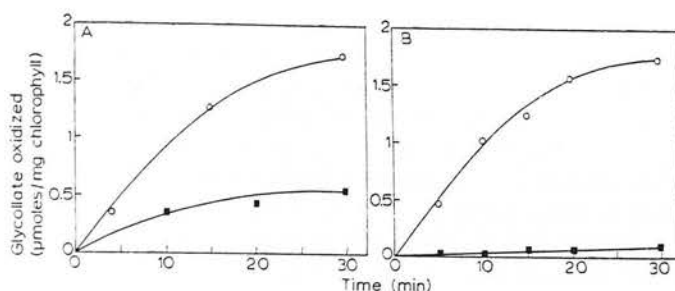


Fig. 1. Glycollate oxidase in 'Arnon' and 'Walker type' chloroplasts. The reaction mixture contained in a final vol. of 1.4 ml the following components, in  $\mu$ moles:  $Mg^{2+}$ , 2.1; potassium phosphate (pH 8.0), 90; chloroplasts or supernatant (by the method of ARNON (A) or WALKER (B)) containing between 50 and 70  $\mu$ g chlorophyll. The reaction was started by addition of 1  $\mu$ mole sodium glycollate. 'Supernatant' refers to the solution remaining after removal of chloroplasts. Glycollate disappearance was determined colorimetrically by the method of CALKINS<sup>32</sup>. ■, chloroplasts; ○, supernatant.

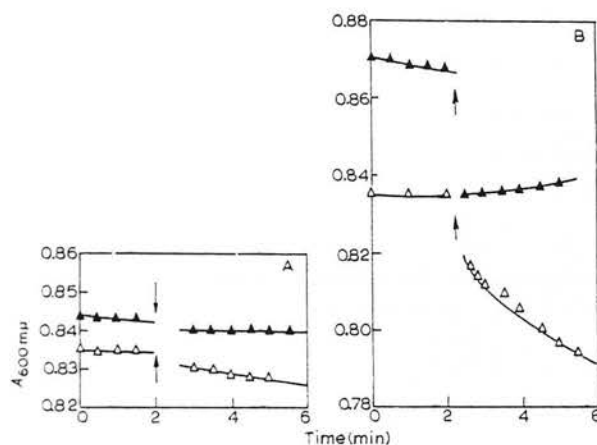


Fig. 2. Glycollate oxidase in chloroplasts from a glycerol gradient. The reaction mixture contained in a final vol. of 2.5 ml the following components, in  $\mu$ moles: potassium phosphate (pH 8.0), 30; DCIP, 0.1; KCN, 10;  $NH_4OH$ , 1; FMN, 0.05; lamellae (A) or chloroplasts (B) containing 21  $\mu$ g chlorophyll. The reaction was started by addition of 20  $\mu$ moles sodium glycollate (indicated by arrows). The decrease in absorbance was measured at 600 m $\mu$  in a Hilger spectrophotometer (ZELITCH AND OCHOA<sup>30</sup>).  $\Delta$ , lamellae or chloroplasts;  $\blacktriangle$ , control (no added glycollate).

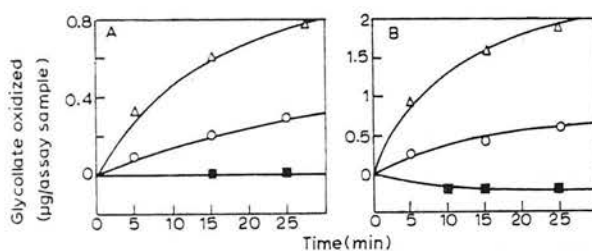


Fig. 3. Retention of glycollate oxidase by chloroplasts. Method as for Table I except that 5 ml glycollate oxidase were used (B). Glycollate disappearance was measured by the method of CALKINS<sup>32</sup>. Chloroplasts from both homogenates were washed in decreasing volumes of grinding medium in order to keep the chlorophyll concentration constant at 50  $\mu$ g chlorophyll per reaction mixture. The control (A) had no added oxidase.  $\Delta$ , unwashed; ○, once washed; ■, twice washed chloroplasts.

the remaining activity. In this way a chloroplast preparation free from oxidase activity could be obtained (Fig. 3). Again, removal of chloroplasts from a tissue homogenate did not alter significantly the specific activity of the supernatant (Fig. 4).

The concentration of cytoplasmic material can be arranged to increase in going down a non-aqueous density gradient. If glycollate oxidase was not chloroplastic then the activity should increase in going down a gradient. (For convenience all non-chloroplastic material will be termed 'cytoplasm'.)

Glycollate oxidase was assayed in successive fractions down a non-aqueous

TABLE I

## GLYCOLLATE OXIDASE ADSORPTION ON CHLOROPLASTS

Alternate right- and left-hand halves were combined from 24 g spinach leaves. The chloroplasts were isolated from both sets of halves by the method of ARNON, WHATLEY AND ALLEN<sup>22</sup> except that the grinding medium for one set contained 8.0 ml of glycollate oxidase (prepared as described under METHODS). Chlorophyll concentrations of each of the four preparations differed slightly and were corrected to an arbitrary value of 10  $\mu$ g chlorophyll per assay sample. Assays as described under Fig. 1.

Fraction	Specific activity ( $\mu$ moles glycollate oxidised/mg protein/h)	
	Homogenate + oxidase	Control homogenate
Chloroplasts	7.1	3.1
Supernatant	22	9.4

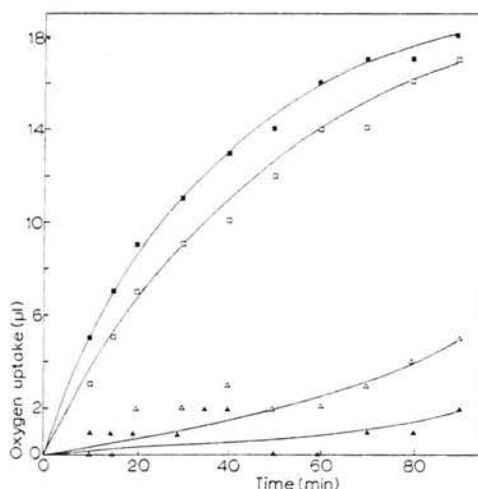


Fig. 4. Comparison of glycollate oxidase activity in whole tissue with that of the supernatant remaining after removal of chloroplasts by the method of ARNON. 100 mg freeze dried spinach leaves were homogenised in 15 ml 0.35 M NaCl in 0.1 M phosphate buffer (pH 8.0) containing 0.0025 M  $Mg^{2+}$ . This suspension was termed whole tissue. Chloroplasts were resuspended in 5 ml 0.1 M phosphate buffer (pH 8.0). Glycollate oxidase was assayed manometrically (CLAGETT, TOLBERT AND BURRIS<sup>31</sup>) in chloroplasts, whole tissue and supernatant. The flasks contained in a final vol. of 3 ml the following components, in  $\mu$ moles: Tris buffer (pH 8.0), 75;  $Mg^{2+}$ , 4.5; 1 ml solution to be assayed and the reaction started by the addition of 20  $\mu$ M glycollate from the side arm. ■, whole tissue containing 120  $\mu$ g chlorophyll/ml; □, supernatant containing 70  $\mu$ g chlorophyll/ml; △, chloroplasts containing 140  $\mu$ g/ml; ▲, whole tissue control (no added glycollate).



TABLE II

## GLYCOLLATE OXIDASE IN FRACTIONS FROM A NON-AQUEOUS DENSITY GRADIENT

Fractions were withdrawn using a Pasteur pipette. Each was diluted with an equal volume of hexane and centrifuged for 10 min at  $3000 \times g$ . The supernatant was poured off and the pellets dried in a vacuum desiccator for 10 min. They were then suspended in 0.05 M phosphate buffer (pH 8.0) and 0.1-ml samples assayed for oxidase activity, as described under Fig. 1.

Fraction	Glycollate ( $\mu$ g) oxidised/fraction
1	0
2	0
3	3.2
4	7.0
5	48.0

TABLE III

## FUMARASE IN DIFFERENT CHLOROPLAST PREPARATIONS

Assays as described under METHODS.

Preparation	Fumarase 240 m $\mu$ (units of absorbance/mg chlorophyll/h)
Non-aqueous chloroplasts	0
Non-aqueous supernatant	13.1
Broken chloroplasts	0
'Arnon type' chloroplasts	
(1) in 0.4 M sucrose	5.2
(2) in 0.35 M NaCl	4.1

TABLE IV

DISTRIBUTION OF COUNTS AFTER [ $1-^{14}C$ ]GLYCOLLATE FEEDING TO WHOLE TISSUE

Leaf segments were fed with sodium [ $1-^{14}C$ ]glycollate and chloroplasts subsequently isolated by the non-aqueous technique. For details see under METHODS. The percentage of the total counts taken up present in chloroplasts was determined by the method of HEBER, PON AND HEBER<sup>39</sup>.

Time of feeding (min)	% chloroplast protein in total protein	Spec. activity (counts/min/mg protein)						Yield of isolated plastids***	% of total counts absorbed present in chloroplasts	
		Chloroplasts		Intact tissue	Chloroplast-depleted tissue	Cytoplasm				
		A*	B**			A*	B**			
5	29.3	1847	1489	2330	1944	2527	2043	8.1	23.0	14.6
15	39	2458	1965	3325	3493	3880	3862	41	28.8	17.0
30	41	2281	1525	3666	3734	4631	4621	11.4	25.5	12.8

\* A, results uncorrected for cytoplasmic contamination.

\*\* B, corrected values assuming that no more than 10% of the total cytoplasmic activity is associated with the chloroplasts.

\*\*\* In % of total chlorophyll content of tissue.

density gradient (Table II). The top two fractions, the upper of which had a relatively high chlorophyll content, had negligible activity. Fractions 3 and 4 had an increasing activity which reached a maximum in Fraction 5. Fumarase activity was taken as a measure of cytoplasmic activity and the distribution of this enzyme in the various fractions followed closely that of glycollic acid oxidase (Table III).

Table IV shows the distribution within the cell of counts taken up when leaf segments were fed with [ $1-^{14}\text{C}$ ]glycollate. From 12.8–17 % of the total counts taken up were found in the chloroplasts of which less than 3 % was present as free glycollate (Table V). [ $^{14}\text{C}_2$ ]Glycollate was not metabolised by chloroplasts isolated by the non-aqueous technique.

TABLE V

DISTRIBUTION OF GLYCOLLATE BETWEEN CHLOROPLASTS AND CYTOPLASM

For details see Table IV and METHODS.

Time of feeding (min)	% chloroplast protein in total protein	Spec. activity (counts/min/mg protein)						Yield of isolated chloroplasts***	% of total counts taken up present as free glycollate in chloroplasts	
		Chloroplasts		Intact tissue	Chloroplast-depleted tissue	Cytoplasm			A	B**
		A*	B			A	B			
5	29.3	695	217	2330	1944	2997	2445	8.1	8.6	2.7
15	39	700	68	3325	3493	5003	4533	41	8.2	0.76

\* Specific activity of chloroplasts expressed here as counts/min in free glycollate/mg protein.

\*\* These figures obtained assuming not more than 5 % of the total cytoplasmic glycollate is associated with the chloroplasts.

\*\*\* As % of total chlorophyll of tissue.

TABLE VI

DISTRIBUTION OF COMPOUNDS LABELLED FOUND IN CHLOROPLASTS AND CHLOROPLAST-DEPLETED CYTOPLASM

For details see under METHODS. Data for each experiment given as % of total metabolised in the fraction; that for total metabolised as  $10^3$  counts/min.

Compound	Chloroplasts		Chloroplast-depleted cytoplasm		
	Time of feeding	5 min	15 min	5 min	15 min
Glycollate	—	—	—	—	—
Glycine	26.4	13.7	33.5	15.1	
Serine	37.6	51.8	33.0	47.5	
Sucrose	7.6	22.3	—	18.6	
Malate	8.0	4.6	3.9	3.6	
Sugar phosphates	20.4	7.5	29.5	15.1	
Insolubles	—	—	—	—	
Total metabolised	1.85	1.87	4.98	5.9	

## DISCUSSION

TOLBERT AND COHAN<sup>8</sup> and DELAVAN AND BENSON<sup>36</sup> have concluded that glycollic acid oxidase is associated with the chloroplasts. While the present work has

shown that some glycollate oxidase is associated with Arnon-type chloroplasts, it has demonstrated that this activity is to be attributed to cytoplasmic contamination and enzyme adsorption on to the chloroplasts.

Fumarase is generally considered to be a mitochondrial enzyme; yet while some activity was present in Arnon-type chloroplasts, none was detected in those prepared according to the method of WALKER<sup>23,24</sup> or by the non-aqueous technique. Part, if not all, of the glycollate oxidase of chloroplasts may be due to contaminating mitochondria.

In contrast to Arnon-type chloroplasts, 'intact' chloroplasts appear to be homogeneous in the light microscope. While some mitochondria are visible in electron micrographs<sup>25</sup> no enzyme activity associated with these could be detected. Such chloroplast preparations are capable of high rates of  $^{14}\text{CO}_2$  fixation (WALKER<sup>27</sup>) suggesting that loss of protein during isolation is small. No glycollate oxidase was found in chloroplasts (50–90 % intact) prepared by this method. Neither was any activity associated with free lamellar systems prepared from a glycerol gradient. However, ZELITCH AND BARBER<sup>17</sup> have reported that malate and glycollate oxidation by isolated chloroplasts was inhibited by addition of 20 % glycerol to the suspension. Thus, while the lack of activity in lamellae may be due to extraction of soluble enzymes, the possibility of some inactivation cannot be ruled out.

More than 25 % of the adsorbed glycollate oxidase could be removed on one washing and the remainder in a second washing. However, ZELITCH AND BARBER<sup>17</sup> using chloroplasts prepared by a method similar to that of ARNON, WHATLEY AND ALLEN<sup>22</sup> found that the addition of glycollate to the washed preparation always gave a marked rise in  $\text{O}_2$  uptake. This oxidase was not removed by as many as 4 washings and it was concluded that some enzyme was present in the chloroplast. It may well be that the lower pH (7.2–7.5) combined with a grinding medium containing mannitol-borate buffer and EDTA used by ZELITCH favoured enzyme adsorption and retention by chloroplasts.

On the other hand these results confirm those of PIERPOINT<sup>19,20</sup> using 'intact' chloroplasts from a sucrose gradient, in which it was concluded that the enzyme was associated neither with chloroplasts nor with mitochondria.

On a non-aqueous density gradient as the density increased, and therefore, the proportion of cytoplasmic components to chloroplasts also increased, so the oxidase activity showed a pronounced increase. The lightest fractions, *i.e.* those containing most chloroplasts, were inactive; the oxidase activity was associated with cytoplasm rather than with chloroplasts. The question of cytoplasmic contamination of the chloroplast fraction need not be considered since no oxidase activity was present in that fraction. There was no reason to believe that any specific inhibition of the enzyme occurred in the chloroplasts since all fractions experienced the same isolation procedure; furthermore added oxidase was not inhibited by the chloroplasts.

Spinach leaf tips readily metabolised externally fed [ $^{14}\text{C}$ ]glycollate *via* glycine, serine and sucrose. Compounds labelled were very similar to those found by MIFLIN, MARKER AND WHITTINGHAM<sup>34</sup> (Table VI).

The results suggest that glyoxalate produced by cytoplasmic oxidation of glycollate enters the chloroplast where it is metabolised to glycine, serine, *etc.* Other compounds formed from glycollate in the cytoplasm may also enter the plastid.

Glycollate has been implicated both in carbon and in hydrogen transport from

the plastid to the cytoplasm<sup>33,30</sup>. Under conditions of low partial pressure of CO<sub>2</sub> glycollate may function as a carbon carrier, replacing sugar phosphates which may be more important under other conditions.

On the other hand, since glyoxalate reductase is confined to the chloroplast and glycollate oxidase to the cytoplasm, the possibility of a glycollate-glyoxalate oxidoreduction cycle operating across the chloroplast membrane is also to be considered. In such a system the pyridine nucleotide of the chloroplast is reduced in light *via* ferredoxin. Glyoxalate within the chloroplast will then be reduced to glycollate by NADPH and glyoxalate reductase. If the glycollic acid is excreted from the chloroplast it could be oxidised externally to glyoxalate and then again re-enter the chloroplast. This mechanism first proposed by ZELITCH AND OCHOA<sup>30</sup> has some of the features of a hydrogen-transport system. The transport of 2 hydrogen atoms from the chloroplast to O<sub>2</sub> in the cytoplasm requires two intermediate hydrogen carriers. The pyridine nucleotide as the initial acceptor can remain in the chloroplast while glycollate and glyoxalate act as 'shuttle' substrates between chloroplasts and cytoplasm.

A similar process may operate in liver mitochondria with  $\alpha$ -hydroxybutyrate and acetoacetate as 'shuttle' substrates (DEVLIN AND BEDELL<sup>38</sup>).

It may be noted that (a) glyoxalate reductase has an equilibrium greatly in favour of glycollate formation<sup>40</sup>; (b) the reverse reaction with glycollate oxidase has not been demonstrated<sup>30</sup>; (c) glycollate movement across the membrane appears to be uni-directional.

Whilst these points are not conclusive, in as far as conversion of glycollate to glycine could still take place within the chloroplast by coupling the conversion of glycollate to glyoxalate with the formation of glycine from glyoxalate, such a mechanism would fail to give a rationale to the observed localisation of the enzyme systems involved.

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## Some Enzymes Present in Marine Mollusca of the Canary Island of Lanzarote

In 1967, a marine biological expedition was organized to the Canary Island of Lanzarote. The main aim of the expedition was to study the benthic ecology of the island using SCUBA diving techniques. Part of the research was aimed at elucidating the feeding habits of the marine mollusca and, to help in this, an investigation was made of some of the digestive enzymes present in the species previously found to be common on the island<sup>1</sup>. Apparently no similar study had been made on most of the species involved<sup>2</sup>. The enzymes studied were:  $\alpha$ -amylase, laminarinase, cellulase, acid phosphatase and acid esterase.

All extracts were made from live molluscs, none of which had been kept longer than overnight following collection from their natural habitat. The normal period between collection and extraction was 4–5 h. The molluscs were

extracted in water by homogenization in a Potter-Elvehjem glass homogenizer. The small molluscs, *Tricolia pullus*, *Rissoa costulata*, *Bittium reticulatum* and *Cantharidus exasperatus* were homogenized in their shells. After homogenization by hand at room temperature for 15 min, insoluble materials were removed by centrifuging in an MSE bench centrifuge. The supernatant, of which the pH was in every case between 7 and 7.4, was decanted and used as the enzyme extract. Apart from *Conus betulinus* and *Aplysia ocellata*, of which single specimens were extracted, at least 5 specimens were used for each result. In the case of the very small molluscs, 50–100 specimens were used.

All enzyme assays were carried out at 26 °C  $\alpha$ -amylase<sup>3</sup>, laminarinase<sup>4</sup>, acid phosphatase<sup>5</sup> and acid esterase<sup>5</sup> were assayed by standard techniques. Cellulase activity was determined by a simplified viscometric technique. To 4 ml of 0.16% carboxymethyl cellulose was added 2 ml of enzyme extract, diluted with water where necessary. After shaking, 5 ml of the mixture was transferred to a U-tube viscometer and the viscosity measured at 10 min intervals. The rate of fall in specific viscosity gives a measure of the enzyme activity.

From the results (Table) it can be seen that all species studied except *Conus mediterraneus* have  $\alpha$ -amylase activity. Cellulase too is present in all species with one exception, *Thais haemastoma*. This may be a reflection of the carnivorous habit of this particular species. Laminarinase is not nearly so widespread but it is at present impossible to place any satisfactory interpretation on its distribution. The same applies to acid phosphatase. The presence of acid esterase in *Cerithium vulgatum* in quantity appears to be unique. Again it is difficult to correlate this with its feeding habit but it may be a fact of some taxonomic interest<sup>6</sup>.

**Résumé.** Les quantités de  $\alpha$ -amylase, laminarinase, cellulase, acide phosphatase et acide esterase ont été estimées par l'examen des extraits de dix-huit espèces de mollusques marins récoltés dans l'île canarienne de Lanzarote.

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Relative activities of digestive enzymes in marine molluscs of Lanzarote

Mollusc	$\alpha$ -Am. <sup>a</sup>	Cell. <sup>b</sup>	Lam. <sup>c</sup>	A.P. <sup>d</sup>	A.E. <sup>e</sup>
<i>Bittium reticulatum</i>	+	?	—	—	—
<i>Cantharidus exasperatus</i>	+	+	?	—	—
<i>Cerithium vulgatum</i>	+++	—	—	+++	++
<i>Conus betulinus</i>	+	++	?	++	—
<i>Conus mediterraneus</i>	—	++	++	++++	—
<i>Haliotis tuberculata</i>	+++	+++	++++	++	—
<i>Littorina striata</i>	++	+++	++	?	—
<i>Monodonta turbinata</i>	+	++	++	?	—
<i>Pusio tricolor</i>	++	+	—	+	—
<i>Rissoa costulata</i>	++	++	++	—	—
<i>Thais haemastoma</i>	++	—	++	+++	—
<i>Tricolia pullus</i>	+++	++	+	+	—
<i>Turbo rugosum</i>	++++	++++	+	++	?
<i>Chiton canariensis</i>	++	+++	++	++	—
<i>Arca lactea</i>	++	+	—	—	—
<i>Pinna rudis</i>	+++	—	++	++	—
<i>Aplysia ocellata</i>	+++	+	?	+	—
<i>Polycera webbia</i>	+	—	—	++	—

+, Trace activity; ++, moderate activity; +++, high activity; +++++, very high activity; —, no activity; ?, slight activity within margin of error.

Value of symbols above

Symbol	$\alpha$ -AM. <sup>a</sup>	Cell. <sup>b</sup>	Lam. <sup>c</sup>	A.P. <sup>d</sup> + A.E. <sup>e</sup>
+	0–5	0–0.5	0–0.05	0–0.5
++	5–15	0.5–2.0	0.05–0.20	0.5–1.0
+++	15–25	2.0–4.0	0.20–0.40	1.0–1.5
++++	Above 25	Above 4.0	Above 0.40	Above 1.5

<sup>a</sup>  $\alpha$ -Amylase (IDC units/g fresh weight). <sup>b</sup> Carboxymethyl cellulase ( $\Delta$  E sp/g fresh weight). <sup>c</sup> Laminarinase (OD Units/g fresh weight). <sup>d</sup> Acid Phosphatase (OD units/g fresh weight). <sup>e</sup> Acid Esterase (OD units/g fresh weight).

<sup>1</sup> J. H. DUFFUS and C. S. JOHNSTON, J. Conch. Paris, in print.

<sup>2</sup> G. OWEN, in *Physiology of Mollusca*, (Ed. K. WILBUR and C. M. YONGE, Academic Press, New York and London 1966), vol. 2, p. 53.

<sup>3</sup> D. E. BRIGGS, J. Inst. Brewing 67, 427 (1961).

<sup>4</sup> A. T. BULL and C. G. C. CHESTERS, Adv. Enzymol. 28, 325 (1966).

<sup>5</sup> K. LINHARDT and K. WALTER, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMAYER; Academic Press, New York and London 1965), p. 779.

<sup>6</sup> We wish to thank Dr. C. S. JOHNSTON and members of the Canary Island Biological Expedition, 1967, for collecting material. We also thank Mr. V. THAMBYRAJAH for carrying out several of the assays. The work was supported by a Royal Society Grant for the purchase of a compressor. One of us (J. H. D.) received a personal grant from the Lord Rootes Memorial Fund. We are grateful to the headmaster of the Instituto at Arrecife for the use of the laboratory there and to Messrs. Bausch and Lomb for the loan of a Spectronic colorimeter.



## NOTES

### Natural Protoplast *Dunaliella* as a Source of Protein

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The protein content of once-washed pellets of *Dunaliella primalecta* was 35 to 48%. Protein quality was good. Electron microscopy confirmed the absence of a cell wall.

The high specific-growth rates of unicellular organisms suggest that they should be capable of considerable productivity. Furthermore, their crude-protein content ( $N \times 6.25$ ) is high (1). However, most algae have a rigid cell wall; the low digestibility and human gastric disturbances associated with the feeding of algae may be caused by its presence. Davies (2) has used mutagens to produce algae lacking cell walls, but their long-term stability was not reported. Preliminary experiments (3) indicate that *Dunaliella* may be suitable for mass cultivation for food. Of particular value in this respect is the absence of a cell wall (4).

The alga was grown in a 20-liter batch culture using filtered seawater enriched with minerals (5). A gas mixture of 5% carbon dioxide in air was bubbled through to maintain carbon dioxide levels over the 2- to 3-week growing period. The light intensity reaching the culture was 10,000 lx, and the temperature was maintained at 30 to 35°C. Algae were harvested using a continuous-action rotor at 18,000 rpm on an MSE high-speed 18 centrifuge and immediately freeze-dried.

Most of the analyses were carried out on once-washed algal pellets. Nitrogen content was determined by micro-Kjeldahl digestion, and crude protein was estimated as  $N \times 6.25$ . Sodium content was determined by the method of Willis (6), and "cellulose" content was measured by the method of Crampton and Maynard (7). Details of the method of protein hydrolysis and the machine used in determining amino acid composition are described by D'Mello (8). Sodium citrate buffers with pH values of 2.75, 2.875, 3.80, and 6.10 placed in a gradient elution device were used.

By washing the algae and removing much of the salt, the percentage of crude protein increased to 52% (Table 1). This compares well

with values reported (1) for other unicellular organisms.

Crude-protein determination is a measure not only of true protein, but also of nonprotein nitrogenous (NPN) substances. These include nucleic acids and other nitrogen-containing molecules. Thus, in *Dunaliella*, about 6% of the crude protein is present as NPN. The amount of true protein present is taken as the sum of amino acid contents after algal protein hydrolysis (Table 2). NPN is then calculated as crude protein (percent) minus true protein (percent). The high growth rates in unicellular organisms are generally associated with high values for NPN. This is due to their relatively high nucleic acid content. For example, the nucleic acid content of *Escherichia coli* (1) is in the range 12 to 16%, and its removal would be required before *E. coli* could be fed to humans. Since *Dunaliella* is unlikely to be fed other than in a mixed diet, and probably not to humans, the value for NPN of around 6%—not all of which is nucleic acid—is unlikely to be a disadvantage.

Electron micrographs of *Dunaliella* (Fig. 1) verify the absence of a cell wall. The small amount of cellulose reported may be associated

TABLE 1. Some nutritional properties of washed and unwashed pellets of *D. primalecta*

Constituent	Unwashed <sup>a</sup>	Washed <sup>a</sup>
Crude protein	41 (3) <sup>b</sup>	52 (6)
NPN <sup>c</sup>		6.2 (2)
"Cellulose"		1.1 (3)
Sodium	6.25 (2)	2.0 (2)

<sup>a</sup> Values are expressed as percentage of dried material.

<sup>b</sup> Number of separate determinations are shown in parentheses.

<sup>c</sup> NPN is taken as crude protein (percent) minus true protein (percent) (see text).

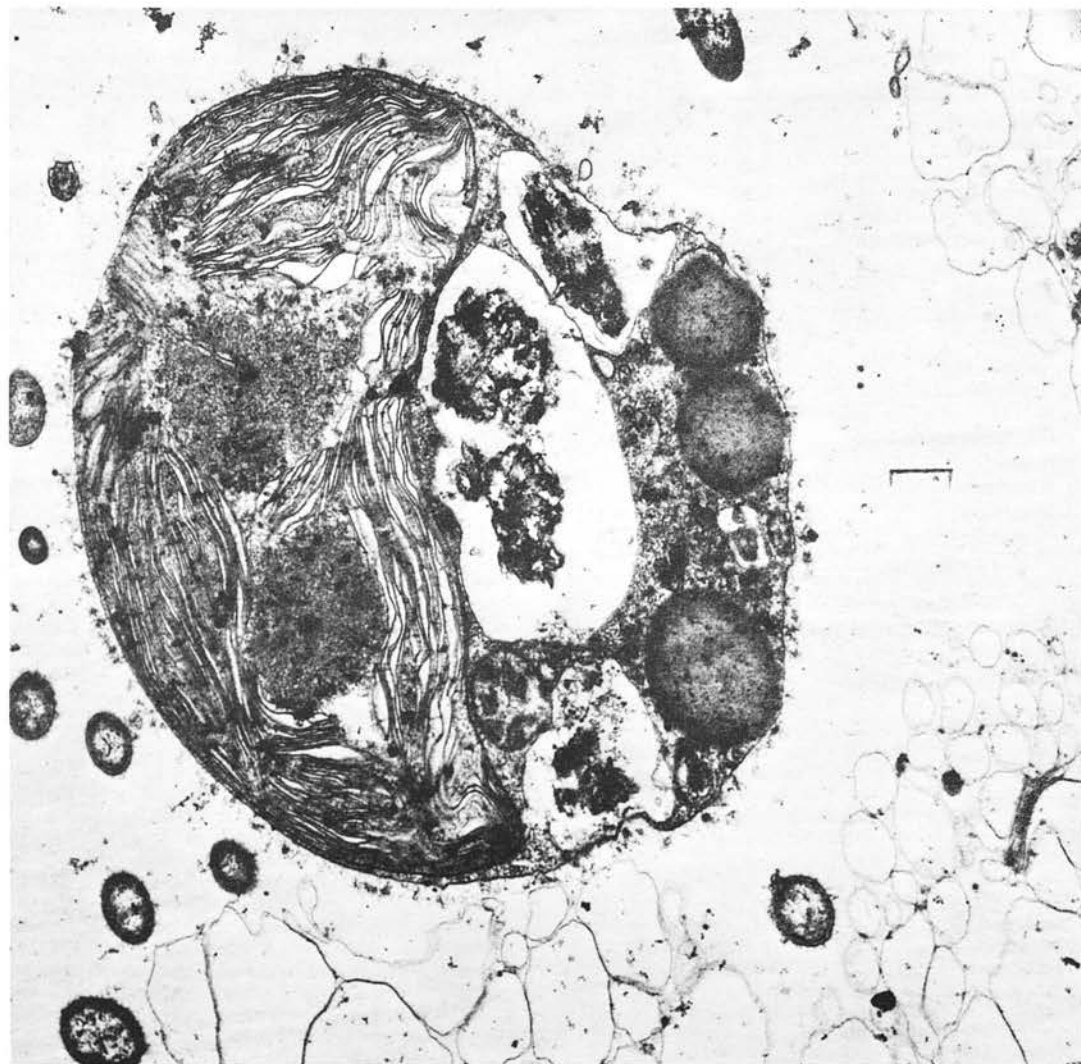


FIG. 1. Electron micrograph of *Dunaliella*. Bar, 1  $\mu$ m.

with the mucilaginous material outside the cell membrane. The sodium levels of the unwashed algae are high. The figure of 6.25% could represent a salt concentration of 16%. Since toxic effects can arise in pigs and poultry at salt levels of above 2%, unwashed *Dunaliella* species could not be fed to these animals at levels above 10% of the diet. Washing removes more than 60% of the sodium and, in theory, algae could then be fed at levels up to 40% of the diet. This problem is eliminated when the alga is used as a food source for young oysters, clams, prawns, and other filter feeders.

The amino acid composition of the algal protein is shown in Table 2. Cells were analyzed from two separate batch cultures—one with a comparatively low protein concentration. When compared on an equal-nitrogen basis, the qual-

ity of the two proteins was very similar. The essential amino acid composition of Food and Agriculture Organization provisional reference protein (9) is shown for comparison. In common with many foodstuffs, including soybean meal, there is a deficiency of the sulfur amino acids cysteine/cystine and methionine. Otherwise, however, the amino acid spectrum is distinctly favorable.

Harvesting can be conveniently done by low-speed centrifugation. Furthermore, since *Dunaliella* precipitates at low temperatures, it may be possible to exploit this as a simpler and more economic method of harvesting.

The evidence suggests, therefore, that *Dunaliella*, with its absence of cell wall and high content of good-quality protein, is a potentially valuable food source.

TABLE 2. Amino acid composition of *Dunaliella* protein

Amino acid <sup>a</sup>	% Dry material (g)		g/16 g of N		
	A <sup>b</sup>	B	A	B	FAO <sup>c</sup> standard
L-Isoleucine	2.82	1.9	5.5	4.3	4.2
L-Leucine	5.73	3.9	11.1	8.9	4.8
L-Lysine	2.71	2.4	5.25	5.5	4.2
L-Phenylalanine	2.80	2.5	5.4	5.7	2.8
L-Tyrosine	1.91	1.6	3.7	3.7	2.8
L-Cysteine/L-cystine	0.40	0.5	0.78	1.13	2.0
L-Methionine	0.99	0.80	1.92	1.8	2.2
L-Threonine	2.82	1.5	5.5	3.4	2.8
L-Tryptophan					1.4
L-Valine	2.88	2.0	5.6	4.6	4.2
L-Cysteic acid	0.19		0.37		
L-Aspartic acid	5.85	3.7	11.3	8.4	
L-Serine	2.44	1.5	4.7	3.4	
L-Glutamic acid	6.02	4.4	11.7	10.1	
Glycine	3.02	2.0	5.8	4.8	
L-Alanine	3.86	3.1	7.5	7.04	
L-Histidine	0.23	0.8	0.47	1.9	
L-Arginine	3.13	2.3	6.10	5.3	

<sup>a</sup> Tryptophan, proline, and hydroxyproline were not analyzed. Performic acid oxidation was not used in cystine determinations.

<sup>b</sup> Values are shown for two separate batches (A and B) of *Dunaliella* containing, respectively, 47.8 and 34.9% protein.

<sup>c</sup> FAO, Food and Agriculture Organization; standard (essential amino acids only) is shown for comparison.

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